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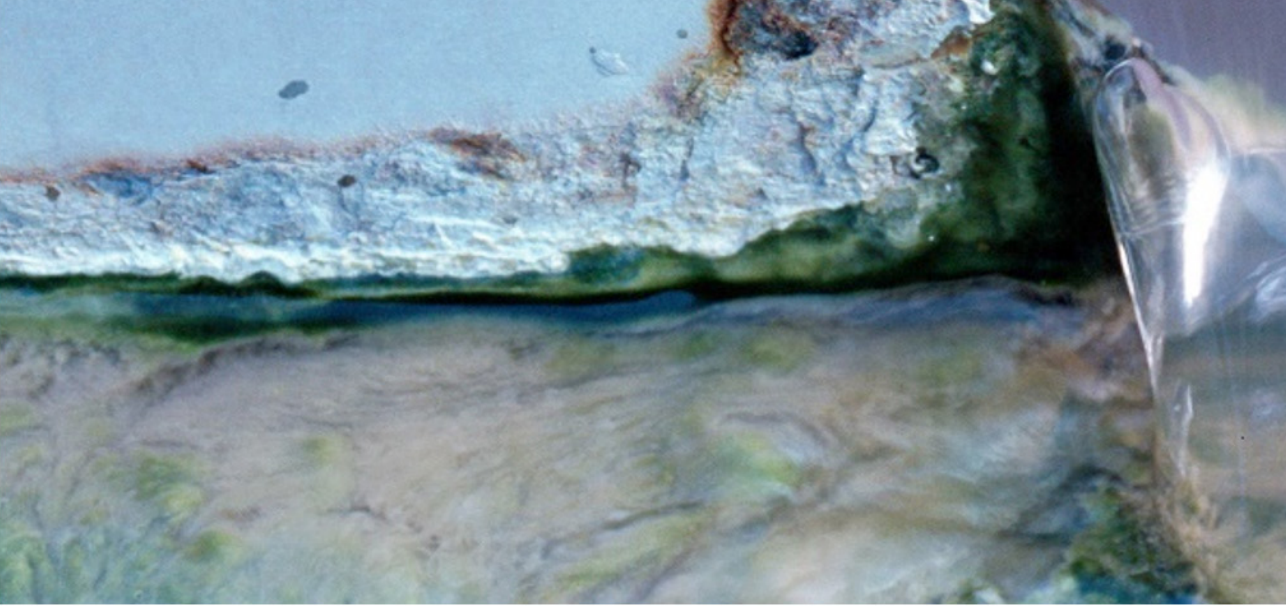

# ELUCIDATING EXOPOLYMERS IN MEMBRANE FOULING

A METAPROTEOMIC APPROACH

**BY**
**SUSAN HOVE HANSEN**

DISSERTATION SUBMITTED 2018

AALBORG UNIVERSITY
DENMARK

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Dissertation submitted April 2018

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# CV

Since I was a little girl, I have always had an intensely inquisitive mind, wanting to get to know how things work and how they are made. In school, I liked physics and chemistry very much, but was truly fascinated when I first learned about DNA and molecular biology. It was then obvious for me to study molecular/biological engineering, and in 2011, I got my Master of Science degree in Biotechnology under the elite programme (cum laude) at Aalborg University. During my Master studies, I got a sniff at cancer research on an internship at the German Cancer Research Center (DKFZ) in Heidelberg, Germany. Back in Aalborg, I specialised in protein chemistry in blood coagulation. Eventually, I ended up in the Environmental Biotechnology Group under Professor Per Halkjær Nielsen studying microbial communities as a research assistant and PhD fellow. Biofilms and "omics" techniques have been my main topics in the context of wastewater treatment and engineering, which I also got the opportunity to study during a research visit at the fine facilities at the Singapore Centre on Environmental and Life Sciences Engineering (SCELSE). Recently, I started as a scientist in the start-up company DNASense, offering customised microbial community analysis.

When I am not dealing with microbes, I am dealing with my own little family, including my robot scientist husband and our 3-year-old daughter and 1-year-old son, who know how to keep me busy.

# ENGLISH SUMMARY

Membrane bioreactors (MBRs) have made their entry into the field of wastewater treatment due to the advantages of high effluent quality and low footprints. However, membrane fouling remains, despite several years' research, the Achilles heel of this promising technology. Fouling is the build-up of unwanted biomass on the membrane that reduces flow, lowers treatment effectiveness, and eventually shortens membrane lifetime. In this PhD project, the problem of fouling has been investigated with focus on the role of activated sludge composition and in particular the proteinous extracellular polymeric compounds (EPSs) components and single microbial cells. The problem has been addressed by interdisciplinary approaches from the field of engineering with modelling and filtration tests in a laboratory scale MBR, combined with approaches from the field of microbial ecology and molecular biotechnology, especially the more sophisticated metaproteomics method, where the total protein content in a given sample is evaluated.

Liquid phase constituents of activated sludge such as colloids, soluble microbial products (SMPs) and EPSs are considered the usual suspects as primary membrane foulants. Their negative impacts were confirmed in this project, however, from microscopical investigations and filtrations of model bacterial suspensions, the planktonic cells in sludge supernatant were emphasised as key foulants. Planktonic cells are often overlooked with standard sludge characteristics measurements, but they can act as possible pioneering organisms with the ability to initiate biofilm formation or so-called biofouling on the membrane surfaces and form severe irreversible fouling.

With the application of molecular methods, it is possible to get a deeper understanding of the individual constituents or properties of the potential foulants in the form of EPSs or SMPs. Novel sequencing techniques enable the retrieval of whole metagenomes from sludge samples (DNA from all microorganisms) and from them recover more or less complete genomes, which are of great importance as they serve as the blueprint of putative functions. Relying on the genomic reference databases, post-genomic methods such as metaproteomics yield information about all the expressed proteins within a sample. In this project, new methods were proposed to use metaproteomics on activated sludge in order to gain useful information on the protein content and potentially link specific EPSs to the producing organism. However, metaproteomics in very complex systems has still not reached its full potential as we still only see the tip of the iceberg of the entire metaproteome.

Amyloids are a type of proteinous EPSs found in activated sludge. They are known to provide robustness to biofilms and are considered an important extracellular matrix compound due to their structural properties, which makes them very interesting in

regard to membrane fouling. With the knowledge of the insoluble nature of amyloids, it was possible to set up a methodology for high-throughput screening. With special sample pretreatment followed by metaproteomics and an automated data processing pipeline, it was possible to detect known amyloids from an activated sludge sample using a known amyloid expressing bacterial strain as standard. By using this method, potential novel amyloid candidates in activated sludge were detected. The method shows great potential, but also challenges in the search for new insoluble foulants.

The amyloids do not only provide structural robustness to biofilms. From binding affinity analyses, it was clear that amyloids have the ability to bind small quorum sensing (QS) signal molecules and most probably play a role in the cell-to-cell communication controlling biofilm formation and EPS synthesis. This potential feature of amyloids to keep QS signalling molecules in check, together with their confirmed structural properties in biofilms, makes them a very interesting target for further research in biofouling in MBRs.

By combining interdisciplinary approaches, this PhD project provided useful insight into the fouling mechanisms in MBRs in wastewater treatment, emphasising the need to encounter the planktonic cells as key foulants of MBR sludge. Methodologies have been developed for applying metaproteomics on MBR biomass, which is the state-of-the-art molecular method for functional investigations of complex biological samples. Last, but not least, a novel function of the amyloidic EPS has been discovered, opening up new possible strategies for fouling control in MBRs.

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# DANSK RESUME

Membran bio-reaktorer (MBR) har vist sig som en meget lovende teknologi indenfor spildevandsrensning. MBR teknologien muliggør etableringen af mere kompakte rensningsanlæg med en væsentlig bedre kvalitet af det rensede vand. For at MBR teknologien kan vinde yderligere indpas, er der specielt én enkelt procesteknisk ulempe der kræver en løsning. Tilstopning af membranerne, såkaldt fouling, er stadig, på trods af flere års forskning, den største hindring for den videre udbredelse af MBR teknologien. Fouling reducerer flowet over membranen og dermed effektiviteten af rensningen og nedsætter i sidste ende membranlevetiden. I dette ph.d. projekt er problemet med fouling blevet undersøgt med fokus på den rolle sammensætningen af aktivt slam spiller. Fokus har været på de proteinholdige ekstracellulære polymeriske stoffer (EPS) samt mikrobielle enkeltceller, der er tilstede i aktivt slam. Problemet er blevet belyst gennem en tværfaglig tilgang: Fra en ingeniørmæssig vinkel med filtreringstest og modellering af data fra en laboratorieskala MBR i kombination med metoder fra mikrobiel økologi over til mere sofistikerede metoder fra molekylær bioteknologi, blandt andet metaproteomics, hvor alle proteiner i en given prøve identificeres ved hjælp af massespektrometri.

De væsentligste komponenter i aktivt slam i forbindelse med fouling befinder sig i den opløste fase. Der er tale om kolloider, opløselige mikrobielle produkter (SMP) samt EPS. Deres negative indvirkning på filtrering er velkendt i litteraturen og det samme blev bekræftet i dette projekt. Nærmere undersøgelser af disse komponenter ved hjælp af mikroskopi og DNA farvning satte fokus på en ofte overset komponent, nemlig de mikrobielle enkeltceller. Filtreringstests af slamsupernatant samt veldefinerede bakterielle opløsninger viste at enkeltcellerne spiller en væsentlig rolle i fouling af membraner. Det er ikke muligt at skelne de mikrobielle enkeltceller fra EPS med standardmetoderne til måling af slamkaraktaristika, derfor påpeger resultaterne fra dette studie vigtigheden af at tage højde for disse enkeltceller. Mikrobielle enkeltceller har desuden potentiale til at danne biofilm på membranoverflader og dermed forårsage alvorlig irreversibel fouling.

Med anvendelse af molekylære metoder er det muligt at opnå en mere detaljeret viden om potentielle slamkomponenter relateret til fouling. Nye sekventeringsteknikker gør det muligt at lave metagenomer fra slamprøver (DNA fra alle mikroorganismer) og fra disse udlede mere eller mindre komplette genomer, som er af stor betydning da de tjener som opskrift for de formodede funktioner. Ved hjælp af genomiske referencedatabaser giver post-genomiske metoder, så som metaproteomics, informationer om alle udtrykte proteiner i en prøve. I dette projekt blev der foreslået nye metoder til at anvende metaproteomics på aktivt slam for at opnå brugbare informationer om proteinindholdet samt potentielt at koble specifikke EPS med de producerende organismer. Imidlertid har anvendelsen af metaproteomics i meget

komplekse systemer endnu ikke nået sit fulde potentiale, da det stadig kun er muligt at se toppen af isbjerget af hele metaproteomet.

Amyloider er ekstracellulære proteiner i form af fibriler, der findes som EPS i aktivt slam. De er kendt for at give robusthed til biofilm og anses som en vigtig bestanddel i den ekstracellulære matrix på grund af deres strukturelle egenskaber, hvilket gør dem særdeles interessante i forhold til fouling af membraner. En metode til screening for proteiner med amyloidlignende egenskaber blev udviklet som en del af dette ph.d. projekt. Baseret på viden om amyloidernes uopløselige egenskaber blev der udviklet en speciel prøveforberedelse som efterfulgtes af metaproteomics og en automatiseret databehandlingsprocedure. Med denne nye metode lykkedes det at påvise kendte amyloider i en kompleks slamprøve samt at finde mulige ukendte amyloidkandidater i aktivt slam. Metoden udviser stort potentiale, men også udfordringer i jagten på nye amyloidlignende slamkomponenter som er interessante i forhold til fouling.

Amyloider giver ikke kun robusthed til biofilm. Som et anden del af dette ph.d. projekt blev amyloidernes potentielle funktioner i en biofilm undersøgt. Ud fra bindingsaffinitetsmålinger blev det påvist at amyloiderne var i stand til at binde til små ”quorum sensing” (QS) molekyler og dermed spiller de med stor sandsynlighed en rolle i bakteriernes celle-til-celle kommunikation og er med til at regulere og kontrollere EPS syntese og biofilmdannelse. Denne potentielle funktion sammen med deres strukturelle egenskaber gør amyloiderne yderligere interessante i forhold til videre forskning i fouling i MBR systemer.

Dette ph.d. projekt har bidraget med værdifuld viden om fouling mekanismerne i MBR systemer. Ud fra tværvidenskabelige samarbejder blev det påvist at mikrobielle enkeltceller spiller en vigtig rolle i fouling af MBR systemer. Derudover har projektet resulteret i udviklingen af nye metoder til anvendelse af metaproteomics til undersøgelse af MBR biomasse. Metaproteomics er den nyeste og mest avancerede metode til at undersøge udtrykte proteiner og dermed funktioner i komplekse biologiske prøver. Sidst, men ikke mindst, har dette ph.d. projekt bidraget med opdagelsen af en ny og spændende funktion af amyloider, som åbner op for nye mulige strategier til at kontrollere fouling i MBR systemer.

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Morten Lykkegaard Christensen and Mads Koustrup Jørgensen also deserve a thank you for being great collaborators from the field of membrane engineering. Thank you for the nice discussions we had trying to minimise the gap between microbiologist and chemical engineers. I think we agreed that the “particles” are alive indeed.

Special thanks go to my great friends and office mates Mikkel, Anja, and Marta who were always ready for a chat - scientific or unscientific. It is a delight to work with you people and to be part of the fun and the good company in the “EB group”.

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*To my beloved mum who saw me start this journey of becoming a researcher and a mother but unfortunately is not among us anymore to see me finish. I know she would have been the proudest woman on earth to be able to put that silly hat on my head. I love you mum!*

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**Paper 2**: Susan Hove Hansen, Allan Stensballe, Per Halkjær Nielsen and Florian-Alexander Herbst. *Metaproteomics: Evaluation of protein extraction from activated sludge*. Proteomics, 14, 2535-2539, (2014) ***Published***

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* These authors have contributed equally to this work.

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# CHAPTER 1. INTRODUCTION

## 1.1. WASTEWATER TREATMENT AND MEMBRANE BIOREACTORS

Cleaning of wastewater plays an important role in maintaining healthy ecosystems in modern societies. Consequently, the release of household or industrial wastewater into water environments eventually causes eutrophication, which leads to annihilation of the ecology of the receiving water. In order to protect the receiving environments, wastewater treatment plants (WWTPs) have been utilised since the early 1900s. Globally, the most widely used wastewater treatment process is the activated sludge process, in which the waste stream is mixed with an "undefined" community of microbes that removes pollutants under controlled conditions (Daigger et al., 2005). The efficiency of the WWTPs depends to a large extent on the function and activity of these microorganisms. The microorganisms in activated sludge can be divided into functional microbial groups according to their roles in the wastewater treatment process. The functions of these groups include the removal of organic matter, nitrogen and phosphorous. In addition, different microbial groups also influence the physical properties of the sludge affecting its ability to settle and be separated from the clean water. Therefore, it is of key importance to maintain a "healthy" microbial community by proper plant operation (Seviour & Nielsen, 2010).

Whereas gravitational separation is used for biomass retention in the conventional activated sludge (CAS) plants, membrane bioreactors (MBRs) - the next generation of environmental biotechnologies - retain the microorganisms by membrane filtration. A schematic overview of the common CAS process versus the MBR setup is shown in Figure 1. The potentials of MBRs are many, and they are already utilised worldwide. One example is the NEWater wastewater reclamation plants in Singapore that are based on membrane technology. By the beginning of 2017, they met 40% of Singapore's total daily water demand (WaterWorld, 2017). Another example is located in the commune of Bassussarry in the department of Pyrénées Atlantiques, France. Here are the greens of the Makila Golf Club lush, green and beautiful all year round. That is all thanks to Bassussarry's population of around 2,400 inhabitants and some innovative wastewater cleaning based on membrane technology (Alfa Laval, 2015).

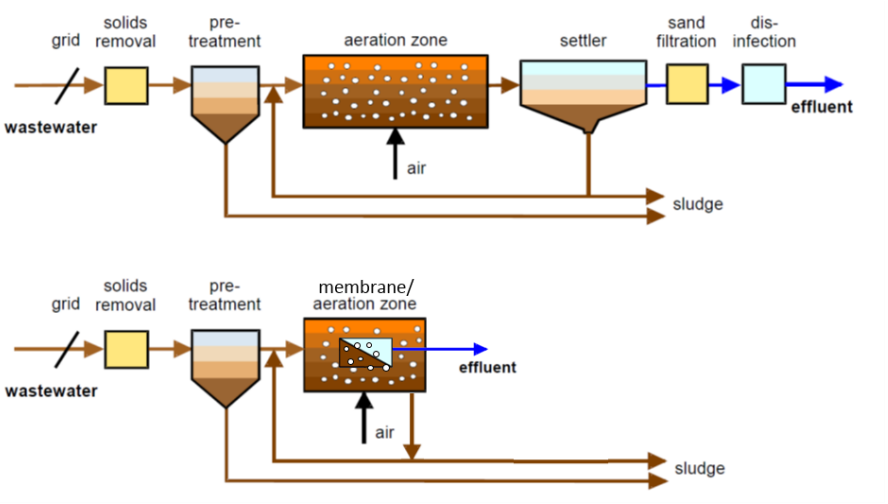


*Figure 1 - Wastewater treatment plant setups: conventional activated sludge process (CAS, top) and membrane bioreactor (MBR, bottom). Modified from Drews & Kraume (2005).*

The advantages offered by MBRs over conventional biological treatment processes are widely recognized as they produce a high quality clarified and largely disinfected permeate product in one single state. MBRs operate at longer sludge retention times, thereby providing the opportunity to select for slow-growing bacterial populations that help take up organic nutrients. Additionally, MBRs reduce sludge production and have smaller footprints (Henze et al., 2008).

The MBRs represent many technical varieties from the type of separation process to the membrane material and membrane configuration. When dealing with activated sludge, microfiltration or ultrafiltration with cut-off values in the range of µm is used for separation of the biomass and the cleaned water (effluent). The membranes used with activated sludge are basically hydrophobic and based on polyvinylidene difluoride (PVDF), because they are cost effective in large scale applications (Judd, 2011). Regarding the configuration, that is the geometry and the way the membrane is mounted and oriented in relation to the flow of water, immersed flat sheet and hollow fibre configurations are the two types to prefer when filtrating activated sludge (Judd, 2011). In 2013, Denmark got its most efficient WWTP regarding effluent quality with MBR technology based on immersed hollow fibre membranes at Mølleåværket in the northern part of Copenhagen (Purac, 2013).

Despite these many advantages, fouling of membranes is the single most important factor that has hampered an even wider application and progression of MBR technology (Daigger et al., 2005). “Fouling” refers to the reduction of membrane permeability due to contamination of the membrane surface (Drews, 2010). It is an inherent property of all membrane-based separations. With activated sludge being a

very dense suspension, with suspended solids content ranging from approximately 3-11 g/L (Jørgensen, Nierychlo, et al., 2017), it is obvious that fouling takes place with deposits of sludge particles dragged onto the membrane surface. Activated sludge is a complex mixture of several different compounds, including sludge flocs, planktonic cells, filaments, extracellular polymeric substances (EPS), dissolved organic molecules (DOM) and salts, which all interact within the MBR reactor and at the membrane surface. Consequently, fouling reduces membrane capacity, leading to increased energy consumption as well as increased need for chemical cleaning and hence shortening membrane lifetime. It also affects the operational costs as higher pressure is needed to maintain membrane capacity and air scouring is required to minimise the deposition of sludge compounds (Daigger et al., 2005). The fouling mechanisms and their causes will be elucidated in more detail in Section 1.3.

## 1.2. AIM

This PhD project was part of The EcoDesign MBR Centre founded by The Danish Council for Strategic Research during the period from 2010-2016. The centre aimed to apply novel approaches in order to design microbial communities in MBRs to strengthen the wider application of MBRs in wastewater treatment. It has been a big collaboration between university sections, several industrial partners and WWTPs. A pilot-scale MBR was built at the local conventional full-scale plant at Aalborg West, Denmark (Appendix A). The pilot plant served as sampling site for MBR activated sludge and was used in different experiments and in many interdisciplinary projects, including this PhD project. In this PhD project, the problem of fouling has been investigated focusing on the role of activated sludge composition with special focus on proteinous EPS components and single microbial cells. The problem has been addressed by interdisciplinary approaches from the field of engineering with modelling and filtration tests in a laboratory scale MBR, combined with approaches from the field of microbial ecology and molecular biotechnology, especially the more sophisticated "omics"-methods. Four main studies were conducted:

- **Study 1** aimed to investigate whether increased levels of bulk planktonic bacteria impair membrane performance due to fouling.
- **Study 2** aimed to evaluate and develop a method to apply metaproteomics on the complex samples of activated sludge for detection of proteins.
- In **study 3**, a specific insoluble proteinous EPS compound (amyloid) was investigated by developing a high throughput method for screening.
- In **study 4**, the specialised functions of amyloids in biofilms were investigated by sophisticated physicochemical molecular methods.

## 1.3. MEMBRANE FOULING

### 1.3.1. FOULING MODEL IN MBRS

Membrane fouling is a very complicated phenomenon and different definitions are proposed in literature (Judd, 2011). However, in this project, a practical definition based on the permeability recovery of the membrane was agreed upon among engineers and microbiologists. The concept is illustrated in Figure 2 and includes the mechanisms of removable, irremovable and irreversible fouling. The removable fouling causes a rapid decrease in the flow of water (flux) through the membrane during filtration. However, after a relaxation period with no filtration, the flux can be restored to a large extent, and therefore this fouling is termed removable. After filtration and relaxation events, the flux does not recover to its previous level, indicating that a type of fouling was not removed by the relaxation and therefore it is termed irremovable fouling. In order to get rid of this irremovable fouling, the membranes are typically exposed to a chemical cleaning procedure. Then, the flux can again be restored to a large extent. Eventually, irreversible fouling is defined as the fouling that cannot be removed by chemical cleaning (Meng et al., 2009). These definitions can also be seen as the observations from the MBR operator's point of view.

From the engineer's point of view, the removable fouling is often ascribed to a cake layer that simply falls off when there is no drag force towards the membrane, whereas the irremovable fouling is known as the gel layer that needs chemical cleaning to be removed (Meng et al., 2009). It can then be discussed if the irreversible fouling must be ascribed to robustness of the gel layer, enabling it to resist the chemical cleaning, or if it is due to long-term corrosion damage of the membrane. Looking into the fouling phenomena through the glasses of a microbiologist, the reversible fouling may

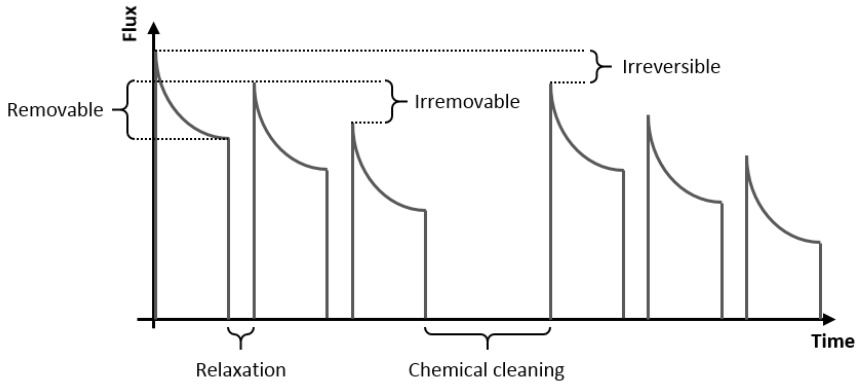


*Figure 2 - The effects of fouling on flux during MBR operation.*

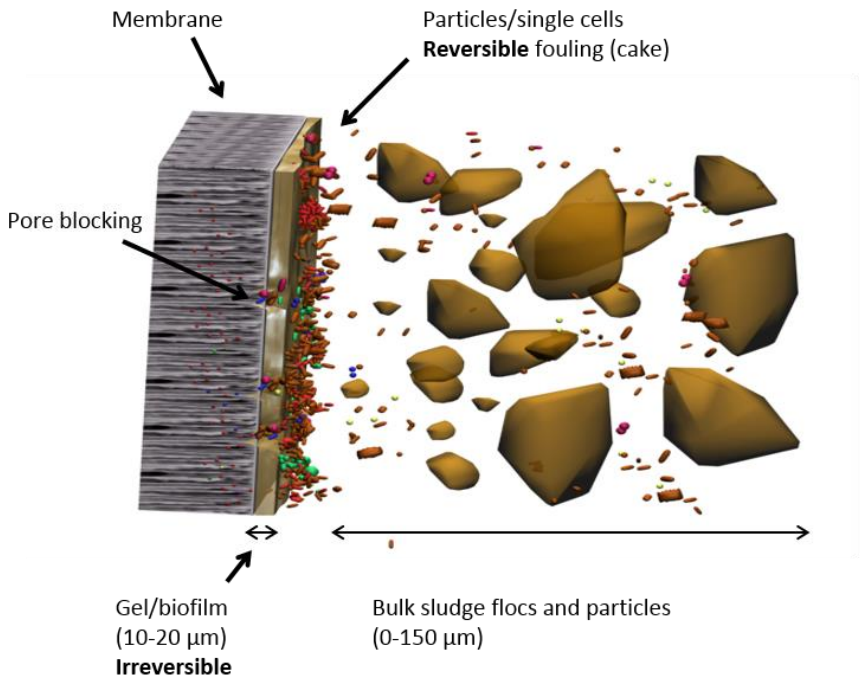


*Figure 3 - Fouling model of activated sludge in MBRs, adapted from Hansen et al. (2015).*

be caused by sludge flocs and suspended solids and irreversible fouling by adhering microorganisms forming a biofilm (Meng et al., 2009). Biofilm formation seems evident because, under initial operation of MBRs colloids, solutes and microbial cells enter the membrane pores and precipitate inside them. During long-term operation when mixed liquor filters through the fouled membrane, nutrients and dissolved oxygen are provided to the deposited bacteria. The bacteria can then multiply and yield extracellular polymeric substances (EPS), thus forming a biofilm, which clogs the pores and forms the strongly attached irremovable fouling layer (Le-Clech, 2010; Meng et al., 2009). A conceptual model of the fouling layers on the membrane is shown in Figure 3. Due to the biological nature of the foulants, the terms biofouling, biocake and biofilm are often interchangeably used in MBR application (Le-Clech, 2010).

### 1.3.2. FRACTIONATION OF SLUDGE

Elucidation of the mechanisms behind fouling layer formation and the fouling properties of MBR sludge is key to be able to develop applicable control strategies in full scale-systems. Fouling propensities and mechanisms can be addressed in many

ways. One strategy is to look at the different sludge fractions and how they each contribute to fouling. From an engineer's perspective, sludge consists of variable amounts of particulate, colloidal and dissolved fractions. The proportions of the fractions can easily be manipulated by operational parameters, for example, shear stress can affect the size of sludge flocs (Wilén et al., 2003). Even a critical mixed liquid suspended solids (MLSS) concentration has been suggested to minimise the presence of smaller particles and enhance filtration (Lousada-Ferreira et al., 2015). A standard procedure has recently been suggested to separate sludge into flocs, colloids and solute fractions (Christensen et al., 2018). Centrifugation is used to separate the flocs and locate the colloids and solutes in the supernatant. Basic physical theories describe how long centrifugation time and speed are needed to separate particles of different densities and sizes. However, the heterogenic nature of sludge flocs makes it difficult to carry out the calculations, therefore, different procedures have previously been suggested in the literature (Itonaga et al., 2004; Su et al., 2008; Wilén et al., 2008).

After harvesting the sludge flocs and other macro structures, the liquid phase of sludge or sludge supernatant remains. Lin et al. (2014) describe the variety of compounds in sludge supernatant, here listed from the smallest to the largest; salts, dissolved organic matters, soluble microbial products (SMP), soluble EPSs and colloids, including organic macromolecules and rigid inorganics such as silica, struvite, and others, but also planktonic single bacterial cells that either came with the influent or detached the sludge flocs. The classification of these liquid phase compounds does not always have a clear-cut boundary. As illustrated in Figure 4, the colloids covering the size range of 1 nm to 10 µm include both some of the SMPs and the suspended single cells present in the sludge supernatant (Lin et al., 2014). In practice, the solutes are isolated from the colloids in a separate fraction, when the sludge supernatant is consequently filtrated, using a pore size of 0.45 µm (Christensen et al., 2018). However, there is a type of particular matter that is covered by both the colloidal and solute fractions, which is the DOMs or so-called "soluble microbial products" (SMPs). As constituents of the bulk sludge phase, SMPs are, as the name says, of microbial origin and can be a product of cell lysis, microbial metabolism or unmetabolised wastewater components. The definition of SMPs is not always clear as in some cases, they are denoted "small microbial products" or even as suspended EPSs (Kraume & Drews, 2010).

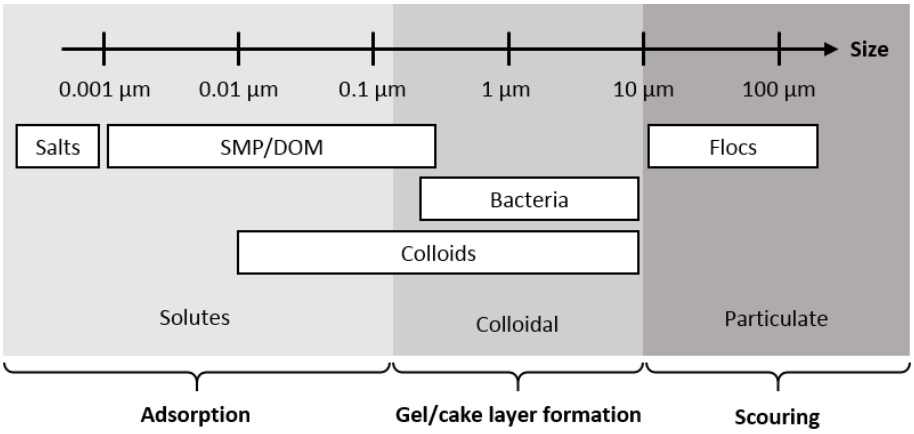


*Figure 4 - Size range of foulants in MBR sludge, adapted from Lin et al. (2014) and Christensen et al. (2018). SMP: soluble microbial products. DOM: dissolved organic molecules.*

### 1.3.3. FOULING PROPENSITY ASSESSMENT

With the sludge fractions defined, theories from physical chemistry and flux models can be applied to assess fouling propensities. Colleagues within the EcoDesign project developed a methodology applying a transmembrane pressure (TMP) step filtration procedure in a laboratory scale membrane module under cross-flow conditions (Jørgensen, Bugge, et al., 2017). After TMP-step filtration, a mathematical flux model is fitted to the experimental flux data to obtain limiting flux, $J_{LIM}$, specific resistance at no pressure, $\alpha_0$, and a compressibility parameter, $P_a$. Equations describing the flux model and the different parameters are formulated in Appendix B. The fouling propensity is described through the limiting flux, where a high limiting flux represents high filterability and is correlated to the critical flux, often used in the literature. This method was applied in a scoping study of 29 Danish WWTPs. It was found that the lower degree of flocculation gives a higher specific resistance and lower back transport (Jørgensen, Nierychlo, et al., 2017). Consequently, it was shown that the level of sludge flocculation was one of the main parameters affecting filtration. An explanation could be that a high degree of flocculation is a result of EPS and colloids being entrapped in the sludge flocs and not present in the liquid phase. Another approach to emphasise the importance of strong flocs in MBR sludge in relation to fouling was taken in Study 1, where the colloid fraction of the sludge was investigated in detail, and a model suspension of planktonic cell cultures was used to study fouling mechanisms. The TMP-step setup was used and demonstrated very high specific resistance of the fouling layers when no flocs were present in the suspensions, thus emphasising the importance of flocs in MBRs. In addition, severe effects of planktonic cells were observed in terms of a fast decline in flux and irreversible

fouling. However, the fouling parameters of separate sludge fractions should be interpreted with precaution. Resistances are usually not additive and use of the resistance-in-series method has been criticised as it neglects coupling or synergetic effects of the different fractions (Chang et al., 2009; Hughes et al., 2006). However, filtration of different sludge fractions can be used to study how different groups of sludge components foul the membrane, and the fractions can be mixed to study how different sludge components interact, as we did in Study 1. This gives valuable information that is difficult to obtain by other methods, making the results more relevant to full-scale studies.

As demonstrated by full-scale surveys, it is among the liquid phase constituents of sludge that the main foulants should be found (Jørgensen, Nierychlo, et al., 2017; Rosenberger et al., 2005). In the literature, it has been argued that EPSs and most significantly SMPs are important foulants and the concentrations of these compounds should be limited to avoid severe fouling of the membranes (Arabi & Nakhla, 2010; Kraume & Drews, 2010; Lin et al., 2014).

The complex phenomenon of membrane fouling in MBRs is becoming unravelled little by little as the effects of sludge properties and process parameters are understood. However, in order to come up with better and more sophisticated strategies to avoid fouling or develop new antifoulants, a deeper understanding of the main foulants, EPSs and SMPs, will be needed. It is well known that EPSs and SMPs consist of polysaccharides, proteins and humic compounds (Flemming & Wingender, 2010a). Nevertheless, information about their individual structures and biological roles in activated sludge and membrane fouling is still limited. The role of EPSs in activated sludge and the way to analyse them will be addressed further in Section 1.4.

## 1.4. BIOFILM LIFESTYLE IN ACTIVATED SLUDGE

In order to address the fouling problems of MBRs, a deeper understanding of the biological content is necessary. Since Costerton's change of paradigm in understanding the bacterial lifestyle in the 1980s, biofilm research has naturally accelerated due to their presence in various ecosystems all over the planet. Biofilms predominate in most of the environmental, industrial and medical problems and processes of interest to microbiologists - with activated sludge as no exception (Costerton et al., 1995). Contrary to the planktonic lifestyle, biofilms have been defined as "aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of EPSs and are adherent to each other and/or a surface" (Flemming et al., 2016). Each biofilm bacterium lives in a customised microniche in a complex microbial community. Within this community, the cells experience primitive homeostasis, a primitive circulatory system and metabolic cooperativity. Each of these sessile cells reacts to its special environment, so that it differs fundamentally from a planktonic cell of the same species (Costerton et al., 1995). Biofilms have high cell densities up to $10^{11}$ cells per gram wet weight and typically comprise many species (Flemming et al., 2016). However, the microorganisms account for less than 10% of the dry mass, whereas the matrix surrounding the cells can account for over 90% (Flemming & Wingender, 2010b). The main component of the matrix is water, which contains the structural and functional components often referred to as EPS that also provide architecture and stability to the biofilm. EPS comprise soluble, gel-forming polysaccharides, proteins, lipids and extracellular DNA (eDNA), as well as insoluble compounds such as amyloids, cellulose, fimbria, pili and flagellae. EPSs immobilise biofilm cells and keep them in close proximity, thus allowing for intense interactions, including cell-cell communication and the formation of synergistic microconsortia (Flemming & Wingender, 2010b). The biofilm properties are summarised in Figure 5.

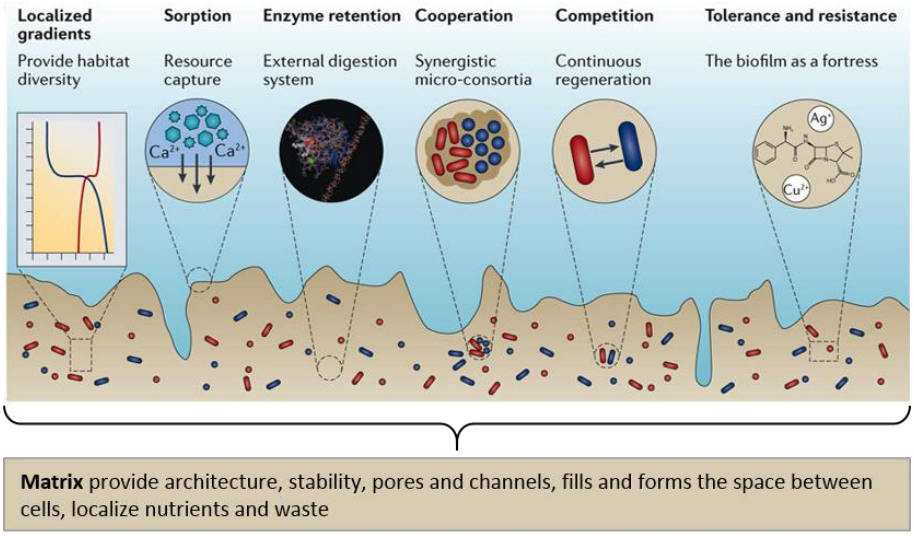


*Figure 5 - Emergent properties of biofilms. From Flemming et al. (2016).*

### 1.4.1. SLUDGE FLOC ARCHITECTURE

The marvellous biofilm behaviour is exploited in the activated sludge process as the bacteria form fluffy biofilms in the form of the so-called sludge flocs with mean sizes of 125 µm in CAS and 65 µm in MBR systems (Christensen et al., 2015). The flocculated biomass can easily be separated from the water phase and the floc size controlled to some extent by operational parameters, such as sludge retention time (SRT) and shear stress. The activated sludge flocs are generally composed of bacterial cells, EPS, adsorbed organic fibres and inorganic fractions. This fractal-like structure of the sludge flocs is kept together by van der Waals and electrostatic forces (DLVO forces), non-DLVO forces (bridging, hydrophobic forces) and physical entanglement (Christensen et al., 2015). The bacteria in activated sludge grow either as single cells, microcolonies, attached growth or filamentous bacteria (Jenkins et al., 2003). The macrostructure of activated sludge flocs is often described with a backbone of filamentous bacteria mostly growing inside the floc, but occasionally sticking out from the floc. Bacteria known as floc formers typically grow as microcolonies or attached growth on the filaments held together by a matrix of EPSs. This structure makes up most flocs with good flocculation, clarification and settling properties in CAS systems (Ekama et al., 1997). In Figure 6, different appearances of activated sludge flocs are visualised as 3D models with corresponding light microscope images.

In addition to the entanglement of inorganic compounds, the floc architecture relies on a fine-tuned interplay between different bacterial species. The microbial

community composition and structure are therefore of great interest in order to link organisms to functions and eventually understand the activated sludge process. The activated sludge ecosystem contains a core community of abundant organisms (Saunders et al., 2016). Information about specific properties of these organisms has been collected in The Microbial Database for Activated Sludge (MiDAS) (McIlroy et al., 2015). The database encompasses a large collection of information from microscopical examination (morphology and Gram type) and ecophysiological analyses (substrate preferences). The MiDAS taxonomy or the systematic classification of wastewater treatment related bacteria is one of the most complete at genus level classification (McIlroy et al., 2015). The MiDAS taxonomy is continuously developed in order to build a common language within the area of microbial ecology in wastewater treatment. This common language is key in order to compare and map the bacterial species with their specific influence on sludge floc properties. Two examples of well-described organisms are *Accumulibacter*, a well-known microcolony former associated with good floc properties, and the filamentous *Chloroflexi* which coincide with poorer flocs and settling properties at high abundances (Bugge et al., 2013). The different floc properties shown by the different species are to some extent a result of their different cell surface properties, but definitely also due to their potential for producing specific EPS. It is beyond doubt that EPSs play a very critical role in the sludge floc architecture. Neutral and charged polysaccharides, proteins (such as amyloids) and eDNA are of special importance to the biofilm architecture in the sludge floc matrix. In particular, the polysaccharides form a hydrated polymer network, mediating the mechanical stability of biofilms often in conjunction with multivalent cations (Flemming & Wingender, 2010a). The cations, such as calcium, create cationic bridges with EPS excreted by the bacteria and thereby hold the various floc constituents together (Wilén et al., 2003).

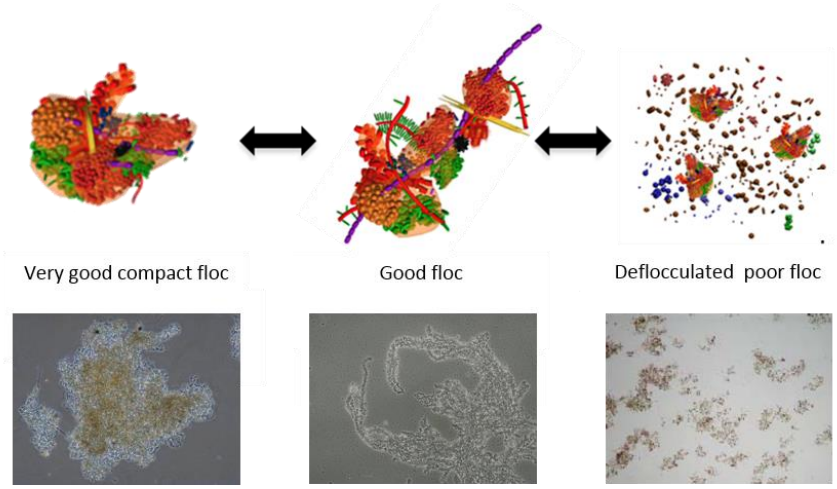


*Figure 6 - Different appearances of activated sludge flocs. Modified from Nielsen et al. (2012) and Perez et al. (2006).*

## 1.4.2. STUDYING EPS

### 1.4.2.1 Visualisation

In order to study the matrix components of biofilms, such as sludge flocs, non-destructive *in situ* techniques are necessary. Microscopy combined with fluorescent dyes has been an important tool to visualise specific EPSs inside biofilms for many years. This type of technique has provided valuable information about the EPSs in activated sludge. Examples are presented in Figure 7, where eDNA is visualised within the sludge flocs with 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) stain and proteinaceous components of the sludge floc matrix, in this case amyloids, are visualised using specific dyes and antibodies (Dominiak et al., 2011; Larsen et al., 2007). Lipid EPSs have been visualised with Nile Red dye inside the sludge floc matrix (Szilveszter et al., 2013) and polysaccharides using special lectins targeting sugar residues (McSwain et al., 2005). Besides verifying the presence of specific EPS, the visualisation tools can also be used for quantification. One example using antibody staining coupled with confocal microscopy indicated that adhesin proteins associated with flagella (FliC), pili (PilA), fimbriae (FimH) and curli (CsgA, CsgB) represented a significant fraction (10-27%) within microbial flocs (Brei, 2012). Another example of quantification of EPSs is in combination with fluorescent *in situ* hybridization (FISH) using gene probes, targeting unique bacterial groups to look for EPS-producing organisms. With this method, Larsen *et al.* estimated the amyloid-producing bacteria to constitute up to 20% of all prokaryotes present in activated sludge biofilms (Larsen et al., 2007).

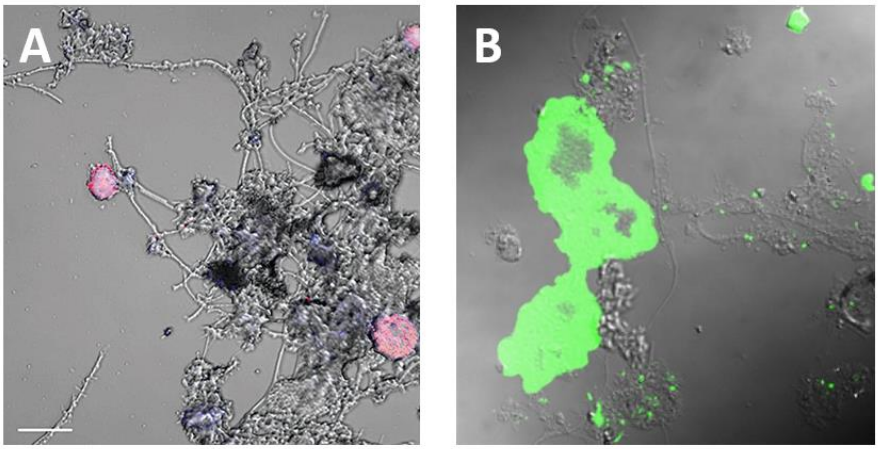


*Figure 7 - Overlays of fluorescent images of activated sludge flocs visualising EPS. A) DDAO staining of eDNA from Dominiak et al. (2011). B) Specific amyloid binding antibody staining from Larsen et al. (2007).*

### 1.4.2.2 EPS extraction and quantification

Other strategies to study the biofilm matrix focus on extraction of the EPSs from the activated sludge flocs. However, efficient EPS isolation is challenging, and there is no universal EPS isolation method; centrifugation, filtration, heating, blending, sonication, treatment with ion exchange resins as well as the use of sodium hydroxide have been reported (Flemming & Wingender, 2010b; Sheng et al., 2010). The most popular method with almost 1700 citations until now was developed by Frølund *et al.* in 1996 and relies on shearing of the sludge with a cation exchange resin (CER). This removes the cations bridging the negatively charged groups of the polysaccharide and protein moieties of EPS (Frølund et al., 1996). Measuring the presence of intracellular enzymes, such as glucose-6-phosphate 1-dehydrogenase (G6PD), as indication of contamination with cellular components, the CER method proved to be the best.

Different methods have also been suggested to distinguish between tightly bound EPS (TB-EPS) and loosely bound EPS (LB-EPS). By using mild and harsh conditions, different molecular weight fingerprints of LB-EPSs and TB-EPSs have been found (Domínguez et al., 2010). However, the separation methodology is not standardised, therefore, the fractions are not defined by their size or nature, in reality, they are a product of a practical procedure. So even though the terminology of LB-EPS and TB-EPS is well established (Lin et al., 2014), it is still used without clear definitions. Another inherent problem with the common EPS isolation techniques is their selection

for water-soluble EPSs, hence losing insoluble EPSs, which could potentially be equally important for the sludge floc properties or foulants.

Conventional chemical colorimetric analyses are today standard to quantify contents of polysaccharides (Anthrone method), protein and humic compounds (Modified Lowry) (Sheng et al., 2010). A recent scoping study of 29 Danish WWTPs found that the average composition of extracted EPS from activated sludge was 42% proteins, 14% carbohydrates and 44% humics (Jørgensen, Nierychlo, et al., 2017). Humic substances are mixtures of compounds that are formed by limited degradation and transformation of dead organic matter and are resistant to complete biodegradation. Additionally, they are very difficult to isolate and very hard to study since they are highly complex, including phenolic and polyaromatic compounds (containing peptide and carbohydrate moieties with carboxylic substituents) (Flemming & Wingender, 2010b). EPSs are very important for the floc properties of the activated sludge, both in terms of quantity and quality. It is known that flocs containing higher concentrations of EPS are generally stronger, however, floc morphology, for example size and amount of filaments, is equally important for the floc stability (Wilén et al., 2003). However, the nature of the EPS seems to play a role, too, as a study found that excessive EPS in the form of LB-EPS could weaken cell attachment and the floc structure, resulting in poor bioflocculation (Li & Yang, 2007).

Many conclusions and correlations have been made from the standardised EPS quantification methods. However, the limitations on relying on colorimetric assays definitely affect the resolution of the information that can be extracted. Other powerful analytical techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared (FTIR) or excitation-emission matrix (EEM) spectrometry have even been used to study fouling in MBRs (Kimura et al., 2015; Ramesh et al., 2007). However, they are much more technically demanding and still only provide the fingerprints of the polymers and give no information on individual constituents or properties of the EPSs or their origin.

#### 1.4.2.3 Using model organisms

Facing the limitations of non-destructive *in situ* techniques to characterising EPS in activated sludge biofilms, pure-culture studies have demonstrated important correlations between EPS and the bacteria of origin. Using molecular methods, it is possible to gain deeper and more sophisticated knowledge about specific EPS molecules, for example, synthesis, regulation and structural information. *Pseudomonas aeruginosa* is a well-studied model organism, which is known to produce unique EPSs. One is an exopolysaccharide (product of the polysaccharide synthesis locus (Psl)) that anchors onto the cell surface and plays an important role in cell-cell interactions and matrix assembly (L. Ma et al., 2009). In addition to the detailed studies of Psl, *Pseudomonas* also produce alginate, thus contributing to the complexity of EPSs produced by a single species. Moreover, an amyloid protein has

been found to be an important EPS produced by *Pseudomonas aeruginosa* (Dueholm et al., 2010). Another well-studied amyloid protein is the curli fibril produced by *Escherichia coli* (Chapman et al., 2002). Both systems are described in detail in the literature regarding biogenesis and structure.

### 1.4.3. AMYLOIDS – SPECIAL PROTEINIOUS EPS IN ACTIVATED SLUDGE

Both the functional amyloids of *Pseudomonas* (Fap) and the curli fibrils have been associated with the activated sludge matrix. As described earlier, antibody staining combined with different microscopic techniques proved the presence of amyloids in activated sludge (Larsen et al., 2007). Moreover, homologous to the curli proteins were found bioinformatically within a metagenome from a wastewater treatment plant (Dueholm et al., 2012), thus making the amyloid a very interesting EPS in biofilm research. The amyloids represent a fascinating class of highly ordered fibrillar protein polymers, which are characterised by a cross-β quaternary structure (Figure 8 A) and the ability to self-assemble from their monomeric counterparts in a nucleation-dependent process (Fändrich, 2007). The Fap production machinery is illustrated in Figure 8 B, showing the operon and the functions of each gene with FapC being the monomeric building block of the fibril. However, due to their insoluble nature and resistance to proteases and denaturants, amyloids are very hard to purify and characterise, therefore, very little is known about their amino acid sequences and structures (Otzen, 2013). Together with Fap and curli, only a few amyloids have been isolated from pure cultures of biofilm-associated bacteria and investigated in depth. However, these amyloids have already provided an amazing demonstration of how amyloids are exploited in biofilms, with roles ranging from fimbriae and other cell appendages used for adhesion and for biofilm formation to structural components of cell envelopes and spore (Blanco et al., 2012; Dueholm et al., 2013). Furthermore, they increase biofilm hydrophobicity and stiffness and thereby make major contributions to the mechanical robustness of biofilms, recognising the amyloid fibres as common building block structures that confer stability to the biofilm matrix (Taglialegna et al., 2016; Zeng et al., 2015).

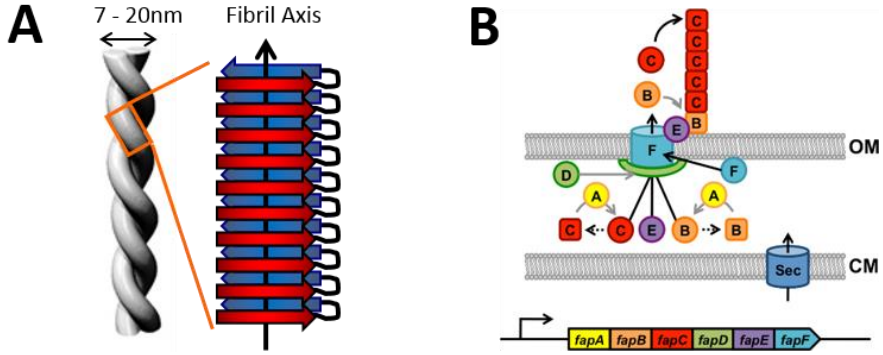


*Figure 8 - A) Structure of the amyloid fibril backbone. B) Fap production machinery, colours indicate genes and corresponding products, outer membrane (OM), cytoplasmic membrane (CM).*

### 1.4.4. MOLECULAR METHODS – POSTGENOMICS AND METAPROTEOMICS

In contrast to pure culture studies, another strategy to link EPSs and microorganisms is the combination of different "-omics" approaches or so-called high throughput techniques. The "meta-omics" techniques enable the analysis of physiological characteristics of whole microbial communities *in situ*. Colleagues at Aalborg University made the first metagenomic study on activated sludge from a WWTP 7 years ago (Albertsen et al., 2011). A metagenome is the DNA from all microorganisms in a given sample and from that it is possible to retrieve more or less complete genomes from the individual microorganisms, which is of great importance as they serve as the blueprint of putative functions (Albertsen et al., 2013). Novel sequencing methods improves drastically these years, and metagenomes of very complex communities such as activated sludge are getting better and better with more closed genomes. Metagenomic information is a prerequisite to carry out postgenomic investigations such as transcriptomics and proteomics. Thereby, availability of genome information for the microbial consortia provides a unique opportunity to potentially characterise the molecular activities and interactions (Hettich et al., 2012). However, only protein-based methods can prove actual gene translation. The term metaproteomics was first used in 2004 and defines "the large scale characterisation of the entire protein complement of environmental microbiota at a given point in time" (Wilmes & Bond, 2004). The same study was the first to apply proteomics on a lab-scale reactor running with activated sludge from wastewater treatment systems. Later, the same authors provided functional evidence of microbial transformations important for enhanced biological phosphorus removal (Wilmes et al., 2008).

### 1.4.4.1 Metaproteomics: Methodology

An overview of the metaproteomic method is presented in Figure 9. The first, easily underestimated, challenge is the extraction of proteins. The protein isolation step is of great importance to the rest of the analysis because a good isolation results in better proteome coverage. However, due to the heterogenic nature of proteins and the complex composition of activated sludge, with interfering substances such as humic substances, the extraction method needed optimisation. Chapter 3 encompasses the paper where we optimised and evaluated protein extraction methods suitable for activated sludge (Hansen et al., 2014). Aiming to reach the highest number of protein identifications and quantitative reproducibility, our study evaluated previously published methods (Barr et al., 2011; Chourey et al., 2010; Kuhn et al., 2011), as well as a commercial soil kit. All extraction methods were different in their use of detergent and cell lysis strategies. The criteria used for comparison of each method included the extracted protein concentration and the number of identified proteins and peptides as well as their phylogenetic origin, cell localisation, functional distribution and quantitative reproducibility. Our results illustrated great differences in output from the various methods, however, we suggested a robust method, which was also less biased towards Gram-negative bacteria. The method is based on special detergents, bead beating and precipitation with strong acid.

The following steps in the metaproteomic method are ensymatic digestion of the proteins and separation of the resulting peptides. Especially the separation process has benefitted from the technical advantages since pioneer studies were conducted using primarily 2D electrophoresis strategies (Wilmes et al., 2008; Wilmes & Bond, 2004). Today, liquid chromatography (LC) is combined with the mass spectrometer (MS), which has accelerated the number of identified proteins from <50 up to 2000 (Hansen et al., 2014).

Following MS analysis, the proteins are bioinformatically identified using reference databases pairing the detected peptide masses to predicted proteins. Software like “Thermo Proteome Discoverer” and “Mascot” is used to process the data together with a database (Hansen et al., 2014). However, handling complex microbial sludge samples, database creation becomes challenging. The public databases such as NCBI are incomplete and sometimes lack proper annotation in the case of activated sludge samples. Furthermore, the huge database sizes can lead to inflated search space, which increases the demand for computing powers and lowers the amount of significant protein identification. To meet these challenges and to decrease false-negative rates, it is of great advantage to use specific metagenomes or 2-step database approaches (Herbst et al., 2016).

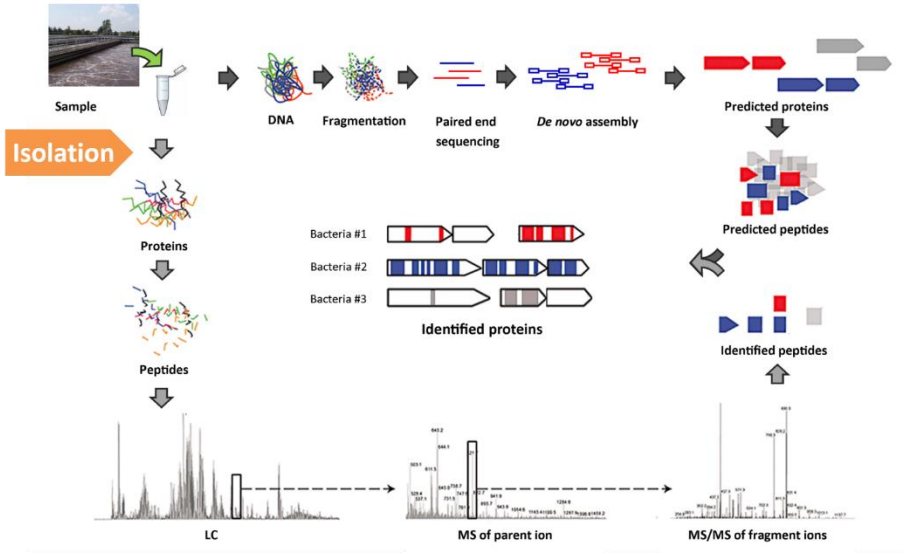


*Figure 9 - Schematic overview of the metaproteomic approach combined with metagenomics. Modified from Denef et al. (2009).*

### 1.4.4.2 Metaproteomics: State of the art

Recently, the metaproteomic approach has been applied in some interesting studies on activated sludge. For example, the sensitivity of metaproteomics has been examined in activated sludge, such that proteins from an added bacterial strain could be detected when this was at around one thousandth of the biomass (Collado et al., 2013). With this sensitivity, it should be possible to target even the less abundant species in the activated sludge communities with metaproteomics. Moreover, the metaproteome of EPS in activated sludge biomass has revealed a number of cytoplasmic proteins, which may play different roles (Wilmes et al., 2015). Most recently, a Chinese group identified around 100 EPS proteins from anaerobic, anoxic and aerobic sludge, respectively, using the CER method prior to metaproteomics. They found that the main roles of extracellular proteins in activated sludge were binding of multivalence cations and organic molecules, catalysis and degradation. They concluded that the structural differences between the different types of sludges could be associated with their different catalytic activities of their proteins (Zhang et al., 2015). In a similar study in Portugal, EPS profiles were also used for comparison of different sludges. Using metaproteomics, it was possible to correlate a group of soluble EPS proteins with longer SRT in a laboratory scale MBR (Silva et al., 2017).

Overall, using metaproteomics in very complex systems such as activated sludge, we still only see the tip of the iceberg. Even for pure cultures, a proteome coverage of only 30 to 70% is achievable. Furthermore, only a small fraction of the theoretical metaproteome that can be inferred from metagenomics is usually identified by metaproteomics. Additional and more sophisticated studies are still needed to determine how much of the total protein biomass is reflected by the identified proteins (Herbst et al., 2016). This should include quantitative studies as well as more refined extraction procedures to look into the structural EPS components of activated sludge.

### 1.4.5. HIGH THROUGHPUT DIRECT IDENTIFICATION OF INSOLUBLE AMYLOID CANDIDATES

As a development to the metaproteomic procedure, a high throughput method for detection of insoluble amyloid-like proteins was developed together with colleagues specialised in amyloid research (Danielsen et al., 2017). The appertaining paper can be seen in its full length in Chapter 4. By taking advantage of the structural stability and the polymeric nature of amyloids, compared to most other proteins, this method utilises formic acid's ability to depolymerise the amyloid fibrils. The procedure is illustrated in Figure 10 and works as follows: (i) The sample is lysed and divided into aliquots that are lyophilised and treated with either 0, 20, 40, 60, 80, or 100% formic acid. (ii) The samples are then lyophilised, dissolved in reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and subjected to short run SDS-PAGE. The amyloid proteins can only enter the gel if they have been pretreated with concentrated formic acid and are therefore only present in these samples. (iii) In-gel digestion is carried out with trypsin and samples analysed by label-free quantitative LC-MS/MS using MaxQuant and the MaxLFQ algorithm. (iv) The data is finally analysed for each individual protein using an automated script and positive amyloid candidates identified on the basis of their abundance profiles with respect to the formic acid concentration.

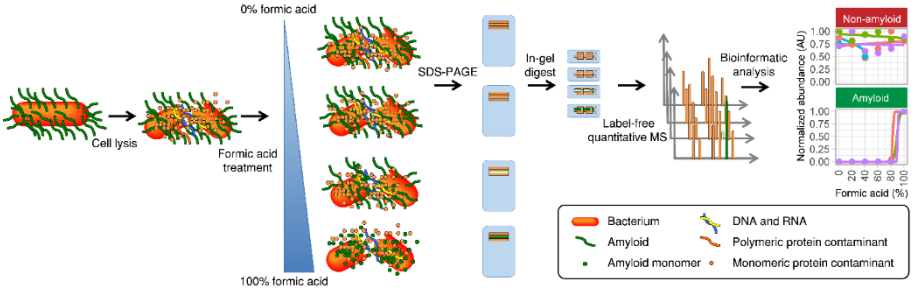


*Figure 10 - Direct identification of amyloids by label-free quantitative MS with formic acid pretreatment. From Danielsen et al. (2017).*

In order to study the potential application of the developed method on complex samples, cells from a known system (10% w/w *E. coli*) were mixed with activated sludge from a Danish WWTP. As expected, the profiles of the CsgA and CsgB monomers from the curli system of *E. coli* still contained the amyloid profile. Interestingly, another structurally confirmed functional amyloid was identified among the sludge proteins, namely the gas vesicle protein A, GvpA (Bayro et al., 2012). Gas vesicles have been found in five phyla of bacteria and two of archaea, most commonly in aquatic microorganisms, and their sequences are highly homologous (Daviso et al., 2013).The characteristic structure of gas vesicles is illustrated in Figure 11. GvpA has not previously been reported in activated sludge, which makes the origin of this functional amyloid very interesting. It must be expected that there are many types of amyloid proteins in different abundances in activated sludge, however, many may not be present at detectable levels using this approach in its current form. Pretreatment steps, like SDS treatment or similar, to remove common and highly abundant globular proteins from the sample may increase the potential identification of amyloid candidates.

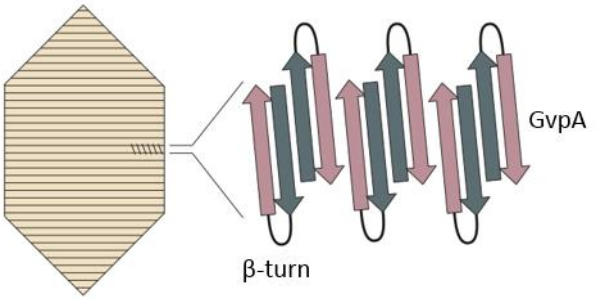


*Figure 11 - Assumed aggregation of gas vesicle particle (GvpA) with the characteristic β-sheets in the ribs of the gas vesicle. From Pfeifer (2012).*

### 1.4.6. AMYLOIDS IN FOULING LAYERS

Fouling layers of MBR membranes would be very interesting to screen for potential novel amyloids with the methodology just described in the previous section. Unpublished results from the EcoDesign project indicated the presence of amyloids in fouling layers from a pilot-scale MBR using thioflavin T (ThT) staining. Despite the fact that ThT stain suffers from some unspecificity towards cellulose and DNA (Ilanchelian & Ramaraj, 2004; Raj & Ramaraj, 2007), it is a good indication that proteinous EPS in the fouling layer are amyloid-like. Another unpublished experiment within this project verified that the modified Lowry assay for standard proteinous EPS concentration determination gave reliable concentrations of purified amyloids when comparing with other assays. However, due to their insoluble nature, they are likely not to be included in standard EPS extractions and therefore not included in EPS concentration determination from sludge presented in the literature. In that sense, the amyloids might simply be overlooked when addressing fouling propensities with standard sludge characterisation methods and proteomics.

In addition to the structural properties provided by amyloids in bacterial biofilms, other specific functions of amyloids were investigated in Study 4. With the use of purified amyloids from overexpressing model organisms followed by structural confirmation experiments, binding affinity analyses and biological reporter assays it was demonstrated that amyloids could be used as reservoirs for quorum-sensing (QS) signalling molecules. Bacteria collectively coordinate their behaviour and communicate with each other using QS systems, whereby small, diffusible molecules are specifically released and then recognised by adjacent cells, where they trigger a response in gene expression (Dietrich et al., 2006). QS signalling systems have been linked to biofilm formation and development by stimulating EPS synthesis (McDougald et al., 2012). When planktonic cells enter the membrane surface in MBRs, it is very likely that QS plays a role in the maturation of the biofouling layer (Vanysacker et al., 2014). The potential feature of amyloids to keep QS signalling molecules in check together with their confirmed structural properties in biofilms makes them a very interesting target for further research in biofouling in MBRs.

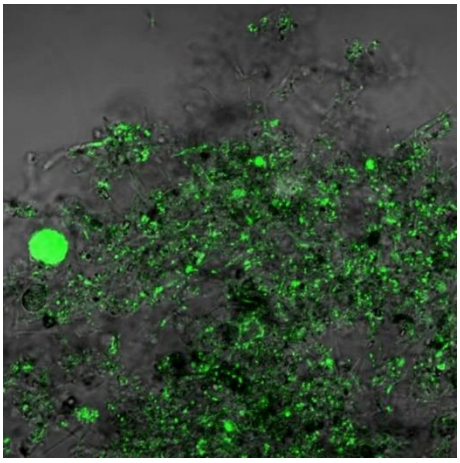

*Figure 12 - ThT staining of fouled membrane from pilot-scale MBR at Aalborg West WWTP showing the presence of amyloids in fouling layer. (Unpublished work by former colleague Poul Larsen.)*

## 1.5. OVERALL DISCUSSION ON FOULING AND MICROBIAL ECOLOGY

As described earlier in Section 1.3.3, there is a general consensus in the literature that the main foulants are found among the liquid-phase constituents of activated sludge. Especially colloids, SMPs and soluble EPS in the bulk sludge should be limited to avoid severe fouling under MBR operation. However, one of the biggest questions that still remains to be enlightened is the problematic biofouling formation process. Does biofilm form from the outside to the inside due to deposition and compression of a cake layer formed by bulk sludge components? Or is it the other way round, due to active growth of pioneer bacteria and biofilm development?

Recent findings suggest that biofilm development or so-called biofouling likely seems to be rather an active process with selective attachment taking place (Vanysacker et al., 2014). In the initial phase of the biofouling formation, differences in species composition between the biofouling layer and the sludge biomass have been found, however, the fouling layers become more similar in terms of bacterial composition to the bulk sludge over time (Gao et al., 2013; Ziegler et al., 2016). These results indicate that planktonic cells originating from disrupted sludge flocs might play a key role in the irreversible fouling layer, however, the effects of planktonic cells originating from the incoming wastewater streams are not yet fully unveiled.

Different physiological characteristics between membrane and sludge isolates have been found, such as higher EPS concentrations and higher ratios of protein to carbohydrate within the EPSs, compared to the sludge isolates (Jinhua et al., 2006; Zhou et al., 2016). However, it is still not clear whether it is protein or carbohydrates that play the biggest role in biofouling. High amounts of carbohydrates in both total and extracted EPS from full-scale activated sludge resulted in more pronounced fouling (Jørgensen, Nierychlo, et al., 2017). The nature of polysaccharides regarding their swelling properties and stickiness makes them plausible components of gel layers in MBRs. However, standard photometric procedures for determination of EPSs in terms of protein and polysaccharide contents have their limitations as described in Section 1.4.2.2, and the full picture of the roles of EPSs in fouling layers is therefore not complete. In order to learn more about the individual constituents or properties of EPSs related to fouling, new methods must be developed or used in combination with the established ones.

The use of metaproteomics for studying proteinous EPSs in fouling layers has not yet reached its full potential. Many household proteins and enzymes have been reported to be present in EPS fractions of activated sludge (Silva et al., 2017; Wilmes et al., 2015; Zhang et al., 2015). However, no structural EPS proteins have been confirmed, nor have proteins involved in extracellular polysaccharide production.

Recently, metagenomic sequencing was used to investigate the microbial structures, functional potentials and biofouling-related genes in membrane bioreactors (J. Ma et al., 2016). According to quantification of the genes associated with EPS biosynthesis, no differences were observed between MBR and CAS systems, and bacteria from the membrane biofilm had lower abundances of genes associated with EPS biosynthesis and transport, compared to the activated sludge in the MBR. Metagenomic results like these give good indications of potential functions, however, only transcriptomic or proteomic data will reveal if the genes of interest are actually expressed.

## 1.6. CONCLUSIONS AND PERSPECTIVES

Combining the research of process engineering and microbial ecology can be a difficult task. However, through interdisciplinary insight, the complexity of biofouling in MBR systems treating wastewater comes closer to becoming unravelled. With a deeper understanding, it is possible to establish better and more sophisticated control strategies in MBR systems.

The negative role of liquid phase constituents of activated sludge in membrane fouling is certain. Likewise, the positive role of the sludge flocs is very well documented. The findings in this project add some more details to this consensus. With Study 1, it was confirmed that the liquid phase constituents of activated sludge had high fouling propensity. Further, it was emphasised to consider the planktonic cells, not only as colloidal particles or SMPs with poor filtration qualities, but also as possible pioneering organisms with the ability to initiate biofilm formation on the membrane surfaces and form severe irreversible fouling.

With the application of molecular methods, it is possible to get information on the individual constituents or properties of the potential foulants in the form of EPSs or SMPs. In the wake of recent years' rapid progression in DNA-sequencing techniques, the field of microbial ecology has entered a new era. Huge amounts of genomic information have been collected in databases, which continue to expand, and new branches of the tree of life are explored. The big focus in recent years, among microbial ecologists within wastewater treatment, is to obtain the genomes of the so far unknown species and fulfil the taxonomic classification of the core community in sludge. Depending on these environment-specific genomic databases, the postgenomic techniques such as transcriptomics and proteomics still lag a few steps behind. With Studies 2 and 3, new methods were proposed to use metaproteomics on complex samples, like activated sludge, in order to gain useful information on the protein content and potentially link specific EPSs to their producing organism. However, metaproteomics in very complex systems has still not reached its full potential as we still only see the tip of the iceberg of the entire metaproteome. In order to improve the results and deductions from metaproteomics, better and more specific protein databases, but also more sophisticated sample processing, are necessary. With the knowledge of the insoluble nature of the specific proteinous EPS, the amyloids, it was possible to set up a methodology for high-throughput screening. The method was validated by detection of known amyloids from an activated sludge sample, where a known amyloid expressing bacterial strain had been added. With this method, potential novel amyloid candidates in activated sludge were detected. The method shows great potential, but also challenges in the search for new insoluble foulants.

Amyloidic compounds are of great interest in biofilm research. Due to their structural properties imparting biofilm robustness, known biosynthesis and their presence in activated sludge, they could most likely play a role in biofouling in MBRs. Study 3

added another possible feature to the functional amyloids from *Pseudomonas* model organisms. From binding affinity analyses, it was clear that amyloids had the ability to bind small QS signal molecules and most probably play a role in the cell-to-cell communication inside biofilms.

The QS system has been a focal point in recent fouling control strategies. Biofilm formation and EPS production are stimulated by QS signal molecules, whereas biofilm detachment is enhanced by QS meditation. Successful application of quorum quenching (QQ) bacteria in terms of biofouling control has been reported in lab as well as pilot-scale. The addition of bacteria that produce QQ enzymes able to degrade specific signalling molecules to laboratory scale MBRs resulted in one third of biofouling (Waheed et al., 2017).

Another fouling control strategy adapted from microbial ecology involves the use of bacteriophages. Bacteriophages have high lytic activity and have been reported to reduce up to 60% of fouling layers (Vanysacker et al., 2014). However, due to their parasitic characteristics, they might not be optimal in full-scale MBRs as the nutrient removal from the wastewater relies on a healthy microbial community. In contrast to the infectious strategy, predators have been suggested for biological fouling control in MBRs. Protozoans, metazoans and nematodes are eukaryotic organisms that are able to feed on bacteria and occur ubiquitously in wastewater treatment facilities. Their presence has been associated with more porous and compressible fouling layers and increased membrane flux in MBRs (Jabornig & Podmirseg, 2015; Klein et al., 2016). However, by grazing on the bacteria, the protozoans and metazoans can also effect the nutrient removal from the wastewater.

Recently, granulation of sludge has been proposed as a strategy to control EPSs and limit fouling in MBRs (Liébana et al., 2018). Utilisation of aerobic granulation resulted in more porous fouling layers and improved membrane flux during filtration. However, granulation of sludge and granule stability is not a simple task and needs special reactor configurations and tightly controlled operational parameters. Other types of granules such as granular activated carbon (GAC) or plastic beads have been applied to achieve fouling control through additional mechanical scouring on the membranes with success (Meng et al., 2017). Even more fouling control strategies are being developed these years, including antifoulants and additives of different types, however, the improvement of membrane performance is not the ultimate and only goal. Strategies to reduce energy consumption and more green/sustainable fouling control ways are very promising areas of future research and development.

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# CHAPTER 2. INVESTIGATION OF FOULING MECHANISMS CAUSED BY FREE CELLS IN CROSS-FLOW MEMBRANE FILTRATION OF ACTIVATED SLUDGE

**Paper 1:**

***Draft manuscript***

Susan Hove Hansen, Mads Koustrup Jørgensen, Rikke Justesen, Per Halkjær Nielsen, and Morten Lykkegaard Christensen. *Investigation of fouling mechanisms caused by free cells in cross-flow membrane filtration of activated sludge*.

# Investigation of fouling mechanisms caused by free cells in cross-flow membrane filtration of activated sludge

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## Abstract

Bacterial consortia in the form of flocs play a key role in wastewater treatment. Membrane bioreactors (MBRs) have made its entry into the field of wastewater treatment due to the advantages of high effluent quality and low footprint. However, in the conventional activated sludge process there is a selection for flocculated bacteria, while dispersed bacteria and particles are removed with the effluent. For MBR sludge, single bacteria are retained by the membrane, so a high content of planktonic cells are often observed. With the current methods, it can be hard to distinguish the colloidal matter in MBR sludge: planktonic cells and soluble extracellular polymeric substances (EPS) or soluble microbial products (SMP). EPS and SMP are considered to be important foulants in MBRs, however, less attention has been drawn to the planktonic cells that immigrates with the wastewater or comes from deflocculation. The aim of this study was to investigate whether increased levels of planktonic bacteria deteriorate the membrane performance and its fouling mechanisms. A submerged flat-sheet membrane laboratory scale set-up was used to test the filtration properties of sludge fractions and model bacterial suspensions. Key filtration parameters were modelled and bacteria were visualised using fluorescent microscopy. Increasing the number of planktonic cells resulted in proportionally higher degree of fouling. Filtration of the bacterial solution and sludge supernatant resulted in poor filtration properties in terms of flux-decrease after a filtration series compared to the flocculated sludge. Whereas, the presence of sludge flocs resulted in more compressible fouling layers and better filterability. We demonstrated that the presence of flocs are very important for membrane performance in MBRs and that particularly the planktonic cells cause problems and might be pioneers for irreversible biofilm formation on the membrane. Future studies should establish methods to ensure good flocculation and floc properties to avoid high numbers of planktonic cells in the MBR sludge.

## Introduction

Membrane bioreactors (MBR) are due to multiple advantages increasingly installed for wastewater treatment around the world. However, membrane fouling remains, despite of several years of research, the Achilles heel of this promising technology as it reduces permeability, membrane lifetime and by elevated pressure and cleaning elevates operating costs. Intensive research efforts have focused on methods to reduce and prevent fouling, including membrane surface modifications, optimization of operation conditions, and cleaning procedures (Le-Clech et al. 2006; Meng et al. 2017).

The characterization of membrane fouling in terms of composition, characteristics, and identification of foulants have achieved lots of attention in the literature. However, bio-fouling mechanisms and the development of so-called biofilm layers on the membrane surfaces are still not fully explored in full-scale

MBR systems (Vanysacker et al. 2014). Therefore, a better and more detailed understanding will be necessary and it should include interdisciplinary research to unify the knowledge from the fields of physical chemistry, process engineering, and microbial ecology to fully realize the potentials and to develop well-adapted control strategies for MBRs in wastewater treatment.

In the conventional activated sludge (CAS) process there is a selection for flocculated bacteria, whereas for MBR sludge, the bacteria are too large to penetrate the pores of the membrane and thus shows a higher content of free cells and soluble EPS compared to CAS sludge (Christensen et al. 2015). Results from MBR case studies have shown a clear relevance of liquid phase constituents, either colloidal or soluble, with regards to membrane fouling (Rosenberger et al. 2005). Additionally, a strong correlation between the degree of flocculation and sludge filterability has been found in full-scale surveys (Bugge et al. 2013; Jørgensen, Nierychlo, et al. 2017). The same was observed by Faust and co-workers who described more severe membrane fouling, the poorer flocs observed based on suspended COD concentrations (Faust et al. 2014). A more controlled laboratory-scale set-up with induced deflocculating affirmed the release of colloidal material that caused severe fouling (Van De Staey et al. 2015). These results indicate that good sludge flocs are important for low level of fouling and that and the constituents in bulk liquid phase of sludge contains the main foulants.

The liquid phase of sludge, or sludge supernatant, contains a variety of compounds. Lin et al. (2014) describe these compounds, here listed from the smallest to the largest; salts, dissolved organic matters, soluble microbial products (SMP) or soluble extracellular polymeric substances (EPS), colloids including organic macromolecules and rigid inorganics such as silica, struvite and others, but also planktonic or single bacterial cells. However, the differentiation between some of the compounds is not very well defined. With overlapping particle sizes, colloids (ranging from 0.01 μm to 10 μm) includes in many cases also SMPs and planktonic bacteria (Lin et al. 2014). This phenomenon might to some extend explain why relative fouling contribution from the sludge supernatant is ranging from 17-90% in different studies (Judd 2011). The denomination of soluble EPSs and SMPs are often intertwined, however, soluble EPS will be used throughout this article. EPSs have often been the usual suspects as membrane foulants (Ramesh et al. 2007) and they have been studied extensively and summarized in the review by Lin et al. (2014).

EPSs are considered to be present in either soluble or in extractable forms. Additionally, the extractable EPS can be considered as light bound or tight bound in the sludge flocs defined by extraction procedures. The main components of EPS are carbohydrates, proteins, humic substances, and nucleic acids (Sheng et al. 2010; Lin et al. 2014). Usually, polysaccharides and proteins are assumed to be the major fractions that contribute to fouling (Drews 2010; Meng et al. 2017). However, the determination of EPS concentration relies almost exclusively on polysaccharide and protein measurements with photometric DuBois (DuBois et al. 1956) or Anthrone (Gaudy 1962) and Lowry (Frølund et al. 1995) assays. These typically used photometric methods give no information on individual constituents, properties, or origin of the EPSs. Neither does alternative methods, such as total organic carbon (TOC) measurements, turbidity, or particle size distributions. Collectively, the listed methods do not quantify the numbers of planktonic cells in sludge suspensions. For example, when total protein is measured, usually with the Lowry method, it should be taken into account that one bacterial cell constitutes with $0.655 \cdot 10^{-13}$ g protein (Frølund et al. 1996; Wilen et al. 2000), meaning that the planktonic cells in most cases are hidden under a surrogate concentration of EPS. Not much attention has been drawn to the planktonic cells in the bulk of the sludge, however, using fluorescent microscopy, Wilén and co-workers showed that the major constituent of the released particles in controlled deflocculation experiments was actually bacteria and floc fragments with only little release of soluble EPS (Wilen et al. 2000). Other powerful analytical techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared (FTIR), or excitation-emission matrix (EEM) spectrometry have been used to study fouling in MBRs (Ramesh et al. 2007; Kimura et al. 2015) but despite of the high-resolution methods, they only give the chemical fingerprints

of protein, humic, polysaccharide, and lipid structures and do not mention a word about single bacterial cells in the fouling layers. In contrast, a recent study of Zhou et al. (2016), combined photometric analysis and EEM with fluorescence and scanning electron microscopy to study foulants and confirmed the presence of various types of bacterial cells. This type of study indicate that many of the previous foulant-characterization studies in MBRs tended to overestimate organic fouling, while the biofouling induced by bacteria may have been overlooked.

Bacteria are similar to colloidal particles in terms of adsorption mechanisms and their physical properties, therefore, colloidal models and theories can be applied to some extend (Eshed et al. 2008). However, their dynamic nature, their mutual interactions, and their capacity to physiologically modify their outer surface in response to environmental conditions are some of the most complicated and often overlooked features of micro/ultrafiltration in MBRs (Vanysacker et al. 2014). To address this gap of knowledge from physical chemical observations to the microbial behaviour on the membrane surfaces, model organisms and methods from microbial ecology should be encountered.

To unravel the complexity of the fouling mechanism and biofilm formation in MBRs, the aim of this study was to investigate whether an increased level of planktonic bacteria could explain the elevated fouling propensity of sludge observed for samples of higher SMP concentrations. This was done by studying how MBR sludge with increasing levels of planktonic bacteria deteriorate membrane performance due to fouling by combining the use of model organisms and sludge characterisation and filterability methods.

# Materials and Methods

## Sludge samples and supernatants

Sludge samples for experimental procedures were collected from Aalborg West Wastewater Treatment Plant (WWTP) with a pilot MBR system running in parallel. The pilot MBR was provided by Alfa Laval A/S and used to treat pre-clarified raw wastewater, treated in a conventional nutrient removing WWTP. The MBR was operated with a membrane module stack (40 $m^2$) of "hollow sheet" PVDF membranes (200 nm nominal pore size, MFP2, Alfa Laval A/S, Denmark). The MBR sludge was collected the day of use and was left for approximately three hours to reach room temperature before characterization and filtration experiments. Filtration experiments with MBR sludge and supernatant were made in sets, so the supernatant was obtained from the same sludge sample that had been stored at 4°C over-night and then processed the following day. The sludge was centrifuged (Sigma 6-16K, Buch&Holm, Osterode am Harz, Germany) at 880×*g* for 2 min to remove large flocs and leave small floc fragments, colloids, and soluble material in the supernatant (Wilén et al. 2008). The supernatant was gently removed with a pipette to avoid disruption of the pellet of sludge flocs.

## Preparation of bacterial cultures

As model bacteria to represent the planktonic cells in sludge supernatant the strain *Pseudomonas* sp. UK4 was chosen. *Pseudomonas* sp. UK4 is a Gram-negative, rod-shaped, bacteria and was originally isolated from a biofilm formed in a drinking water reservoir in a random search for bacteria producing functional amyloids (Larsen et al. 2007). Growth medium for the bacteria *Pseudomonas* sp. UK4 were colony factor antigen (CFA) medium (10 g $L^{-1}$ hydrolyzed casein, 50 mg $L^{-1}$ $MgSO_4$, 5 mg $L^{-1}$ $MnCl_2$, 1.5 g $L^{-1}$ yeast extract, pH 7.4 in double distilled water). Incubation was at 25°C and 200 rpm. Bacterial colonies from CFA-agar plates were transferred to 10 mL CFA medium in 50 mL centrifuge tubes and grown overnight. A volume of 1 L of CFA medium was inoculated with 1 mL of starter culture and grown overnight. The cells were harvested by centrifugation at 5000×*g* for 15 minutes and resuspended in Phosphate Buffered Saline 0.0067 M($PO_4$) (PBS) (HyClone, Thermo Scientific) and stored at 4°C to prevent further growth. Before usage, the samples were homogenized in a glass tissue grinder (Thomas Scientific).

### Dry matter, protein, humus, and polysaccharide concentration

Sludge total solids concentration (TS) was measured in duplicate according to Standard Methods (APHA et al. 2005). One supernatant suspension was analyzed for protein and humic substances according to a modified Lowry method with use of BSA (Fraktion V, AppliChem, Darmstadt) as standard for protein and HA (Janssen Chimica, Geel, Belgium) as standard for humic substances. (Frølund et al. 1995). The concentration of carbohydrates was determined by a modified Anthrone method (Gaudy 1962; Raunkjær et al. 1994) with D(+)-glucose (BDH, Poole, England) as standard.

### Cell counting

In order to count planktonic cells in sludge and bacterial suspensions, a specific fluorescent DNA stain was used. The bacteria were visualised using DAPI (4′,6′-diamino-2-phenylindoledihydrochloride-dilactate) staining. DAPI was applied to a final concentration of 0.05 mg/ml for 5 min. All dilutions were performed in filtered tap water. Homogenized samples for enumeration were filtered onto 0.22-µm-pore-size white polycarbonate membrane filters (Millipore, Bedford, Mass.) and fixed on microscopic slides. Total counts were determined by counting no fewer than 10 microscopic fields.

### Submerged flat sheet membrane laboratory scale reactor set-up

The filtration system was a flat sheet system with aerated membranes, the so-called Aalborg Filtration Property Analyzer (AaFPA) which was previously described by Jørgensen, Bugge, et al. (2017). The total volume of the reactor was 5 L and the active membrane area was 84 $cm^2$ of an Alfa Laval MFP2 flat sheet membrane. The airflow was kept constant at 7.5 L $min^{-1}$ and the temperature was kept stable at around 22°C. The permeate flux was calculated by weighing the permeate on line using a highly sensitive scale and dividing with the membrane area. The transmembrane pressure was adjustable and controlled by the water level difference between the bioreactor and the permeate beaker.

### Sludge fouling propensity

Fouling propensity was determined in two different filtration experiments. First by 1 hour filtration at fixed TMP of 5 kPa (similar to the pilot-scale MBR plant at Aalborg West WWTP from where the sludge was obtained). Between each test, the membrane unit was cleaned with sodium hypochlorite (soaked in 1000 ppm solution for at least 2 hours). The data obtained was fitted to a mathematical model using Equation 1

$$J = J_{SS} + k \cdot e^{-bt} \quad (1)$$

where $J_{SS}$ is the steady state flux, $k$ is the maximum decrease in flux, and $b$ is a rate constant for the flux decrease (Cheryan 1998). A high $b$ value represent a fast reach of $J_{SS}$ whereas a high $k$ value indicate a big loss in flux from $J_0$ to $J_{SS}$. $J_{SS}$ is reached by minimizing the root mean square error (RMSE) between the experimental flux and the calculated flux, by adjusting the constants $k$, $b$ and $J_{SS}$, using the Microsoft Excel problem solver function.

Secondly, TMP-step filtration experiments were conducted to study specific fouling layer resistance, fouling layer compression, and irreversible fouling. The filtration experiments were conducted at 1 hour steps of TMPs varying from 1 to 13 kPa with 2 kPa increments. Between each step, a 1 hour relaxation step was applied to remove fouling. Another mathematical flux model was fitted to the experimental flux data to obtain limiting flux, $J_{LIM}$, specific resistance at no pressure, $\alpha_0$, and a compressibility parameter, $P_a$, as described in (Jørgensen, Bugge, et al. 2017). A high limiting flux represents low fouling propensity, as it is a measure of back transport of foulants away from the membrane due to air scouring. $\alpha_{5000}$ is the specific resistance of a fouling layer at 5000 Pa and will be used to compare specific resistance of different fouling layers. $P_a$ is the compressive strength and inversely proportional to compressibility, as it is the pressure required to double the specific resistance.

# Results and discussion

## Characteristics of sludge, supernatant, and bacterial suspension

Different suspensions of sludge used for filtration experiments was taken from a well working pilot-scale MBR after several months of operation. The characteristics of the suspensions are listed in Table 1. After centrifugation of the MBR sludge, the amount of total solids in the supernatant was reduced to 15% of the original suspension and the pH increased slightly by 0.1. The number of planktonic cells present in the supernatant was $2.49 \cdot 10^7$ cells per mL. The number is higher than typically found in CAS supernatant of $0.25 \cdot 10^7$ cells per mL along with total cell counts in the sludge of $129 \cdot 10^7$ cells per mL (Wilen et al. 2000). This was expected as MBR sludge in general is considered to show a higher content of single cells, compared to CAS sludge (Christensen et al. 2015). Looking into the protein concentration of the sludge supernatant, it can be calculated that bacterial cells constitute almost 20% of the total protein content by multiplication of the total cell count with $0.655 \cdot 10^{-13}$ g protein (protein contribution from one bacterial cell according to Frølund et al. (1996)). This number confirms the importance of direct cell counts when interpreting EPS data. As shown on the micrographs in Figure 1 not only planktonic single cells remained in the sludge supernatant but also some bacteria that were present in the form of filamentous organisms, counting additionally $1.55 \cdot 10^7$ cells per mL.

A bacterial model suspension of the Gram-negative *Pseudomonas* sp. UK4 was cultured in the lab and suspended in nutrient free buffer to avoid proliferation and to keep pH constant in a range similar to sludge supernatant. Bacterial model suspensions were then made with fixed cell numbers by dilution with the buffer. Despite of the similar numbers of planktonic cells, difference in turbidity was observed between the sludge supernatant and the bacterial model suspension, which most probably is due to the filamentous bacteria but also other colloids present in the sludge supernatant. Similar to the turbidity values, TS is higher of the sludge supernatant compared to the bacterial model suspension. Again, this may be attributed to the filamentous types of bacteria but also the inorganics present in the sludge supernatants. Despite of the presence of filamentous bacteria in the sludge supernatant, they will not be considered further in this study, as the focus will be on the planktonic single cells and their fouling propensities due to their colloidal nature. The bacterial strain chosen for the model suspension *Pseudomonas* sp. UK4 belongs to the family of Pseudomonadaceae, which has been reported to be abundant in wastewater influent (Saunders et al. 2016). The strain *Pseudomonas* sp. UK4 forms rod-shaped cells with a size of 1 µm as visualised in the micrographs in Figure 1, and it can form biofilms (Larsen et al. 2007). It should be noticed that not all cells in this bacterial model suspension are single or planktonic, as some are found in small aggregates after homogenization procedures, however, the aggregates are not near the range of sludge flocs (65-125 µm) (Christensen et al. 2015). By using this model planktonic cell suspension, it is possible to mimic the conditions in the MBR plants and study the effect of increasing levels of incoming planktonic cells that are present in full-scale plants due to immigration from the wastewater.

***Table 1*** *- Characteristics of sludge, supernatant, and bacterial suspensions.*

| Suspension | TS (g $L^{-1}$) | pH | Cell count (cells $mL^{-1}$) | Turbidity ($OD_{650nm}$) | Protein (mg $L^{-1}$) | Humics (mg $L^{-1}$) | Carbohydrates (mg $L^{-1}$) |
|---|---|---|---|---|---|---|---|
| Bulk sludge | 9.6 | 7.2 | - | 0.034 | - | - | - |
| Supernatant | 2.0 | 7.3 | $2.49 \cdot 10^7$* | 0.026 | 14.58 | 14.28 | 5.4 |
| Model bacteria | 0.01 | 7.4 | $2.74 \cdot 10^7$ | 0.006 | - | - | - |

*the cell count corresponds to the planktonic single cells, however, additional $1.55 \cdot 10^7$ cells were present as filaments.

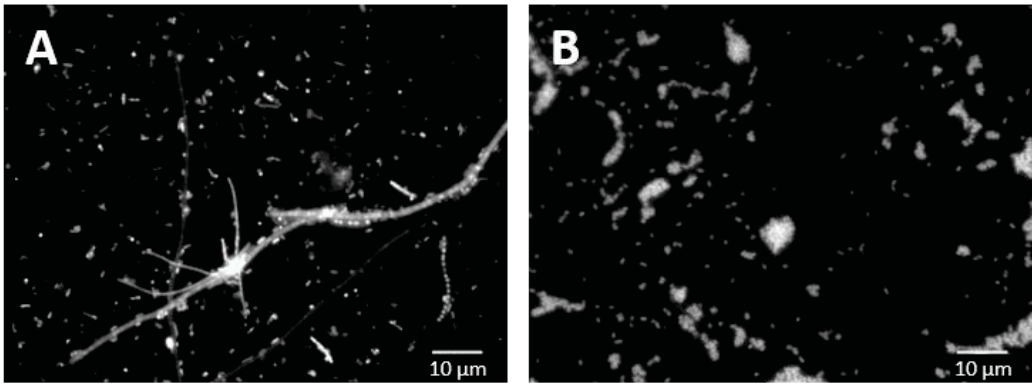


***Figure 1*** *– Micrographs of DNA stained bacteria from A) MBR sludge supernatant. Many free cells in the size range of 1 µm and some filamentous bacteria. B) Pure laboratory culture of a rod-shaped bacteria used in filtration tests (Pseudomonas* UK4*).*

## Fouling propensities of single cells

In order to evaluate the effects of increasing numbers of planktonic single cells one hour filtration experiments at fixed TMP of 5 kPa, simulating the pilot-scale MBR from where the sludge originated, was performed. Flux development of bulk sludge, sludge supernatant and model bacterial suspensions with increasing number of cells was monitored and resulted in a fast initial decline in flux followed by an equalization of flux at the end. The rate of flux decline varied depending on the suspension that was filtered, see Figure 2. A model (Equation 1) was fitted to the experimental flux data and the model profiles and the rate constant *b* for the flux decrease were used for the comparisons. All model parameters are presented in Table S1 in supplementary. As observed from Figure 2B, the rate constant *b* increased with higher numbers of planktonic cells resulting in more rapid flux decrease. The linear coherence between the filtration rate constant *b* and the number of planktonic cells indicates that there was a direct correlation between concentration and rate of fouling, which can be a result of higher convective drag of cells to the membrane surface. The rate of flux decline and planktonic cells count for sludge supernatant and sludge supernatant with planktonic cells were in line and follow the trends of the model suspensions, indicating that the cell count in supernatant determined the rate of flux decline.

The addition of 4 times more planktonic cells from the model bacterial suspension clearly affected the filtration profiles of sludge and supernatant as a faster flux decrease was observed for both sludge and supernatant when the number of planktonic cells were quadrupled (Figure 2B). By comparing the rate constant *b* for the flux decrease, the effect of bacterial addition seems to be even more pronounced for the sludge than for the supernatant. The positive effects of sludge flocs on filterability has previously been described in full-scale surveys (Rosenberger et al. 2005; Jørgensen, Nierychlo, et al. 2017), however, it was not observed in this short-term filtration experiments. Another parameter, the steady state flux $J_{SS}$ also seemed to correlate with the number of planktonic cells; the more cells, the lower $J_{SS}$. This was confirmed by the modelled value of $J_{SS}$ but also the approximated value from the experimental data (Table S1). For the supernatant, $J_{SS}$ also decreased after addition of the model bacteria, whereas for the bulk sludge, the opposite was observed. This might be due to adsorption of planktonic cells to sludge flocs over time. However, even though the addition of 4 times more planktonic cells to the bulk sludge did not decrease $J_{SS}$, it definitely led to a faster drop in flux during initial filtration.

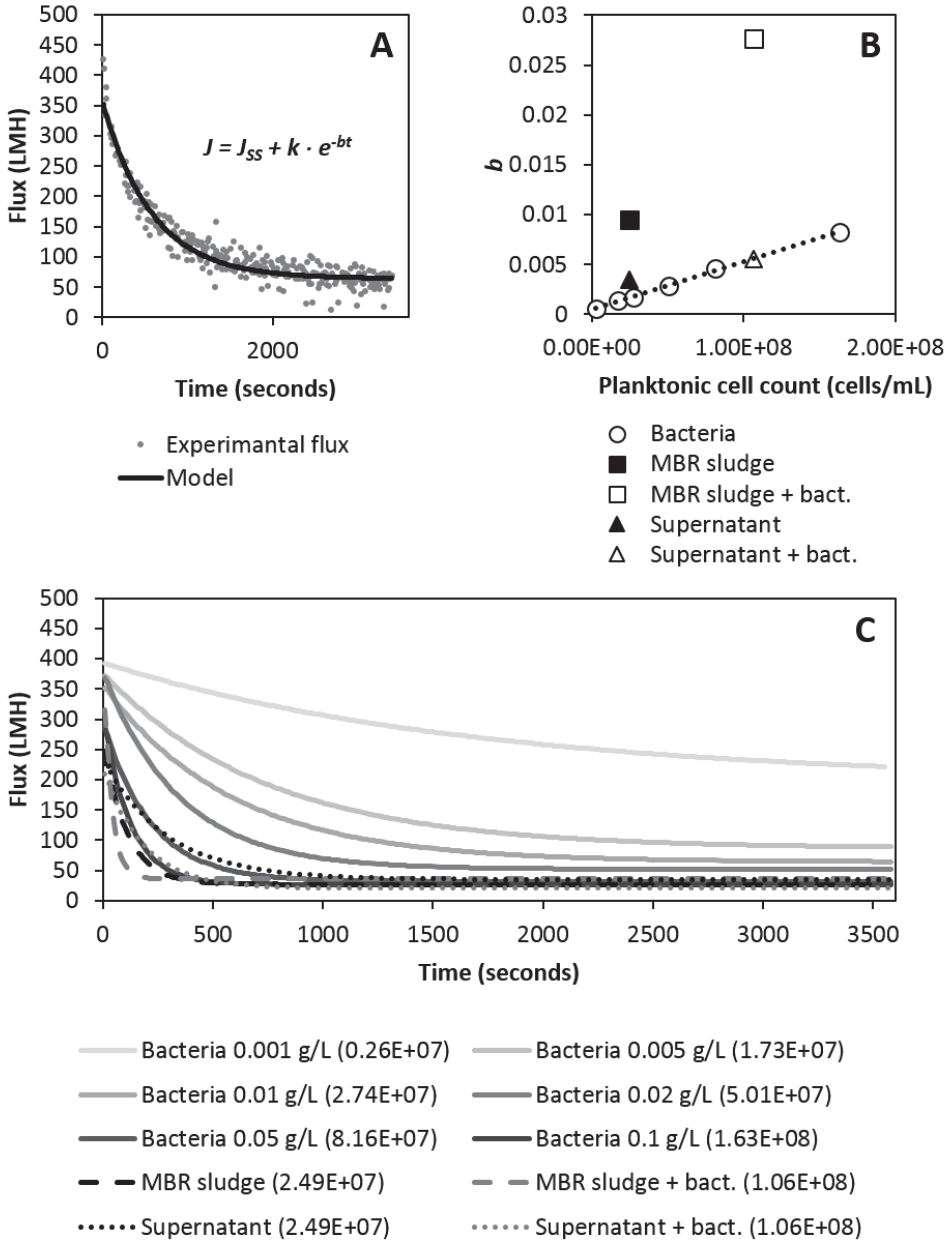


***Figure 2*** *- Filtration experiments. A) Experimental flux data from 1-hour filtration of bacterial suspension (0.01 g $L^{-1}$ and 2.74 · $10^7$ cells $mL^{-1}$) and fitted model. B) Plot of rate constant from the model versus number of planktonic cells in suspension. C) Modelled flux data from 1-hour filtration of different suspensions of planktonic bacteria and modified sludges. Number in parenthesis denotes planktonic cells $mL^{-1}$.*

**Irreversible fouling caused by planktonic cells**

To link fouling propensities to the planktonic single cells TMP step test filtration series was performed on bulk sludge, sludge supernatant, and a bacterial model suspension with similar single cell count as the sludge supernatant. Experimental flux data from the TMP step test filtration series was obtained from the three different suspensions, Figure 3. After fitting the flux model described previously by Jørgensen, Bugge, et al. (2017), filtration parameters was determined, see Table 2. It was clear from the results that the model works for "sludge-like" suspensions that form compressible cakes on the membrane, and that the bacterial suspension did not show the same behaviour. Furthermore, the model takes the dry matter concentration into account, which was very low in the model bacterial suspension. The values obtained for the bacterial suspension should therefore be interpreted with care. However, it was very clear that $J_{LIM}$ from the step test filtration of sludge was almost twice of that from the supernatant, representing a higher filterability of the sludge and indicating the beneficial roles of the sludge flocs.

A recent survey of 29 different sludges from various types of pilot and full-scale wastewater treatment plants in Denmark, using the same step test model to estimate the specific resistance at 5000 Pa of the fouling layer presented values ranging from $0.45 \cdot 10^{12}$ to $8.4 \cdot 10^{12}$ m kg$^{-1}$ (Jørgensen, Nierychlo, et al. 2017). The sludge used in this study from a pilot-scale MBR plant had similar filtration properties with a specific resistance of $1.1 \cdot 10^{12}$ m kg$^{-1}$. Previous studies have demonstrated that cake resistance is highly dependent on SMP in sludge supernatant (Wang et al. 2014). However, due to different determination methods, it is hard to compare directly to the values obtained for the supernatant and the bacterial suspension in this study. For extracted EPS, specific resistances have previously been reported to be of the order of $10^{14}$ up to $10^{17}$ m kg$^{-1}$ (Wang et al. 2009; Nagaoka et al. 1996). Assuming that the bacteria in the model suspension are rigid spheres of equivalent diameters of 0.5 µm a specific resistance of $6.75 \cdot 10^{12}$ was predicted from the Carman-Kozeny equation. However, the modelled specific resistance from the filtration data was $2.51 \cdot 10^{14}$ for the bacterial model suspension, indicating that the bacterial cells are compressible like previously observed for yeast cells (Meireles et al. 2002).

The most compressible fouling layer was formed during the step filtration of bulk sludge. The beneficial role of sludge flocs was clearly demonstrated as $P_a$ increased by a magnitude of six when filtering supernatant compared to bulk sludge, resulting in a less compressible fouling layer formed from sludge supernatant. Similar results were also confirmed by dead-end filtration by Poorasgari et al. (2015). The picture is even worse for the bacterial suspension, as would be expected due to the homogeneity of the suspension lacking filaments and macromolecular structures.

The percentage decline in flux ($\Delta J$) from first to last filtration at 1 kPa in the TMP step filtrations, listed in Table 2, revealed significant formation of irreversible fouling ranging from 15-19% for supernatant and bulk sludge, to 61% for planktonic cells. This demonstrates the severe effects of the planktonic cells on membrane performance. Similar effects were seen by Hassan and co-workers who added smaller (1-2.5µm) *Escherichia coli* bacterial cells to a filtration of yeast, which are larger cells, and observed severe irreversible fouling (Hassan et al. 2013). The bacterial cells might cause pore blocking or adsorb to the membrane, but also the formation of a biofilm, which are not easily removed by air scouring or during relaxation, could be an explanation to the irreversible fouling. This study, along with many others, demonstrates the severe fouling effects of colloidal and particular matter in the sludge supernatant (Rosenberger et al. 2005; Lin et al. 2014). However, where many studies rely on particle detection or extractable EPS measured by colorimetric assays (that may or may not include single cells), this study directs the attention to the planktonic cells. Therefore, focusing on a well-defined bacterial suspension this study were able to eliminate the other particular matter thus highlight the effects of the planktonic single cells.

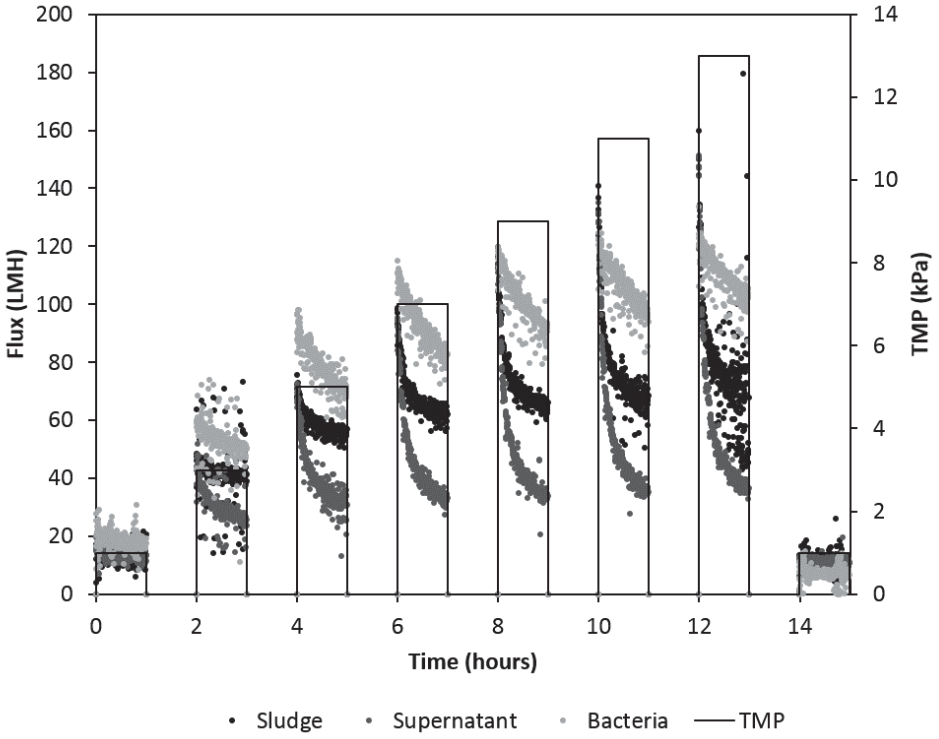


***Figure 3*** *– Experimental flux data from TMP step test filtration of bulk MBR sludge, supernatant, and model bacterial suspension.*

***Table 2*** *- Filtration parameters from TMP step test modelling*

| Suspension | $J_{LIM}$ (LMH) | $\alpha_0$ ($m\ kg^{-1}$) | $P_\alpha$ (Pa) | $\alpha_{(5000\ Pa)}$ ($m\ kg^{-1}$) | RMSE | $\Delta J$* |
|---|---|---|---|---|---|---|
| Bulk sludge | 70.5 | $8.63 \cdot 10^{10}$ | 423 | $1.11 \cdot 10^{12}$ | 29.18 | 19 % |
| Supernatant | 36.6 | $6.71 \cdot 10^{12}$ | 2523 | $2.00 \cdot 10^{13}$ | 16.75 | 15 % |
| Model bacteria | 101.1 | $2.51 \cdot 10^{14}$ | 60048 | $2.11 \cdot 10^{14}$ | 22.75 | 61 % |

** $\Delta J$ is the decrease in mean flux in percentage from the first filtration at 1 kPa and the last filtration at 1 kPa after the TMP step series.*

**Planktonic cells in MBRs**

Eventually, the planktonic cells will be absorbed on the membrane surface due to their size and behaviour in the membrane concentration polarization layer (Christensen et al. 2018). The planktonic cells enters the MBRs through the wastewater influent, and their fate is partly unknown. Some might be adsorbed in the sludge flocs and some might end up in fouling layers. Microbiological analyses of fouling layers indicate the presence of pioneer species, however, the fouling layers becomes more similar in terms of bacterial composition to the bulk sludge over time (Gao et al. 2013; Ziegler et al. 2016). Planktonic cells originating from disrupted sludge flocs might therefore also be of special interest as they might play a key role in the irreversible fouling layer. Most microorganisms are able to produce EPS and form a biofilm on the membrane after colonisation. Some bacterial strains and populations show higher biofouling potential than others. For example *E. coli* possessed higher biofouling capacity compared to *Staphylococcus epidermis*, and *Flavobacterium lutescens,* despite their somehow similar morphology (size and shape) (Lee et al. 2010). Another recent study found that the fouling potential of bacteria are highly strain dependent and found some features shared by key fouling-causing bacteria including formation of convex colonies with swollen podgy shapes and smooth lustrous surfaces with high water, hydrophilic organic matter and carbohydrate content (Ishizaki et al. 2016). The irreversible biofouling of microfiltration membranes may be more highly correlated to specific bacterial populations rather than the total, bulk concentration of biomass but it remains to be investigated.

**Biofilms and fouling**

The low compressibility and high resistance of irreversible biofouling layers like the one obtained in this study from filtration of the bacterial suspension might be related to biofilm properties. In addition to the effects of the closely packed cells, the bacteria undergo adaptation to their life in a biofilm context by producing a matrix of EPS that definitely also play a key role in strength of the biofouling layer (Flemming & Wingender 2010; Vanysacker et al. 2014). Especially the carbohydrate EPSs have been considered important in terms of fouling. High amounts of carbohydrates in both total and extracted EPS from full-scale activated sludge resulted in more pronounced fouling (Jørgensen, Nierychlo, et al. 2017). Additionally, very high filtration resistance has been obtained when studying the isolated effect of polysaccharides by filtration of agarose (Chen et al. 2016). The “sticky” effects of polysaccharides have likewise been demonstrated by the sludge flocs ability to reflocculate after cellulase treatment (Xie et al. 2010). In the TMP step filtration experiment, no substrate was added to the suspensions, however, with a duration of 15 hours it cannot be precluded that a biofilm on the membrane surface was supplied with dead or lysed bacterial cell debris which then could be used as a food source. Without evidence from microscopy, we cannot confirm the presence of a typical biofilm after the filtration series, but it is very likely as the *Pseudomonas* bacterial strain previously has been isolated from a biofilm (Larsen et al. 2007). Likewise, other members of the *Pseudomonas* genera are considered biofilm formers and some have the ability to produce alginate, which is a well-known extracellular polysaccharide and structural component of biofilms (Colvin et al. 2012). Strategies to avoid severe biofouling in MBRs might therefore focus on mitigating biofilm attachment and maturation on the membrane itself but also limit the numbers of planktonic cells in the liquid phase of the activated sludge maintaining good strong flocs. However, it seems like a two-edged sword on one side aiming to mitigate biofilm formation on the membrane surface and on the other side aiming to enhance the biofilm strength of the sludge flocs.

All of these observations suggest that the adhesion of bacterial cells is much more complicated than the adhesion of colloids and that it depends on several factors. Moreover, it is difficult to extrapolate the results from monospecies experiment to real MBR systems, but these findings should be integrated and considered in the development of control strategies.

# Conclusions

This study aimed to describe the influence of planktonic single cells on sludge fouling propensity. The planktonic cells are most often hidden under a surrogate concentration of EPS or encountered as colloidal particles from turbidity measurements and particle size distributions. Based on direct cell counts it was found that the planktonic bacterial cells constitute almost 20% of the total protein content of the liquid phase constituents of sludge. The effect of the cells was significant, as filtration tests showed that fouling was governed by the amount of free cells in the bulk liquid, as the cell count was directly propotional to rate of fouling by cell deposition. Filtration of a bacterial model suspension resulted in less compressible fouling layers with higher resistance compared to bulk sludge. In addition, severe irreversible fouling was observed after a filtration series. Implicit, this indicates the positive role of sludge flocs on membrane fouling. Therefore, it is important to have good sludge floc properties ensuring that bacterial cells are kept in flocs rather than in the supernatant.

Standard sludge characterization measurements including turbidity, particle size distribution, and EPS concentrations do not give the full picture whether colloidal foulants are indeed planktonic cells or EPSs. With this study, we would like to emphasize the need to encounter the planktonic cells, not only as colloidal particles but also as possible pioneering organisms that can initiate biofilm formation on the membrane surfaces and form severe irreversible fouling.

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# Supplementary

**Table S1** – Filtration model parameters from 1 hour filtration experiments at TMP of 5 kPa.

| Suspension | TS ($g\ L^{-1}$) | Cell count* ($cells\ mL^{-1}$) | $k$ | $b$ | $J_{SS}$ | RMSE | $\Delta J_{(after\ 55min)}$** |
|---|---|---|---|---|---|---|---|
| Model bacteria | 0.001 | $0.26 \cdot 10^7$ | 196.3 | 0.00058 | 196.7 | 16.58 | 224 |
| Model bacteria | 0.005 | $1.73 \cdot 10^7$ | 289.2 | 0.00135 | 86.7 | 19.37 | 83.9 |
| Model bacteria | 0.01 | $2.74 \cdot 10^7$ | 293.3 | 0.00172 | 64.2 | 18.10 | 58.8 |
| Model bacteria | 0.02 | $5.01 \cdot 10^7$ | 326.7 | 0.0029 | 52.0 | 19.65 | 42.3 |
| Model bacteria | 0.05 | $8.16 \cdot 10^7$ | 265.2 | 0.00464 | 32.1 | 17.44 | 24.6 |
| Model bacteria | 0.1 | $16.3 \cdot 10^7$ | 285.7 | 0.00823 | 26.8 | 16.03 | 20.8 |
| Supernatant | 2 | $2.49 \cdot 10^7$ | 199.4 | 0.00346 | 35.0 | 16.00 | 29.2 |
| Supernatant + bact. | 2.05 | $10.6 \cdot 10^7$ | 201.0 | 0.00556 | 20.5 | 15.35 | 15.9 |
| Bulk sludge | 9.6 | $2.49 \cdot 10^7$ | 243.4 | 0.00947 | 27.1 | 16.30 | 25.9 |
| Bulk sludge + bact. | 9.65 | $10.6 \cdot 10^7$ | 378.8 | 0.02769 | 36.3 | 15.02 | 33.3 |

*Planktonic cells only

**$\Delta J_{(after\ 55min)}$ denotes the average flux during the last 5 min of filtration approximating the observed $J_{SS}$

# CHAPTER 3. METAPROTEOMICS: EVALUATION OF PROTEIN EXTRACTION FROM ACTIVATED SLUDGE

**Paper 2:**

This research was originally published in *Proteomics.*

Susan Hove Hansen, Allan Stensballe, Per Halkjær Nielsen, and Florian-Alexander Herbst. *Metaproteomics: Evaluation of protein extraction from activated sludge*. Proteomics, 14, 2535-2539, (2014)**.**

TECHNICAL BRIEF

# Metaproteomics: Evaluation of protein extraction from activated sludge

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Metaproteomic studies of full-scale activated sludge systems require reproducible protein extraction methods. A systematic evaluation of three different extractions protocols, each in combination with three different methods of cell lysis, and a commercial kit were evaluated. Criteria used for comparison of each method included the extracted protein concentration and the number of identified proteins and peptides as well as their phylogenetic, cell localization and functional distribution and quantitative reproducibility. Furthermore, the advantage of using specific metagenomes and a 2-step database approach was illustrated. The results recommend a protocol for protein extraction from activated sludge based on the protein extraction reagent B-Per and bead beating. The data have been deposited to the ProteomeXchange with identifier PXD000862 (http://proteomecentral.proteomexchange.org/dataset/PXD000862).

**Keywords:**
Activated sludge / Complex microbial communities / LC-MS/MS / Metaproteomics / Microbiology / Protein extraction

Additional supporting information may be found in the online version of this article at the publisher's web-site

The term metaproteomics was first used in 2004 to describe a proteomic study of a lab-scale reactor running with activated sludge from wastewater treatment systems. However, no extensive metaproteomic studies of full-scale activated sludge have been reported, primarily due to lack of representative reference genomes and metagenomes for reliable identification of the proteins [1]. Fortunately, the availability of metagenomic sequences [1, 2] and the increasing number of reference genomes likely enable the successful application of postgenomic techniques such as metaproteomics. The first step in a metaproteome study is the comprehensive isolation of the entire protein complement of a given sample. Efficient protein isolation protocols focused on laboratory scale sludge bioreactors [3,4] or are optimized for 2DE [5]. Activated sludge has many similarities to soil in terms of e.g. humus content, where extraction procedures for metaproteomics have been well optimized [6]. Aiming to reach the highest number of protein identifications and quantitative reproducibility, this study evaluated two previously published methods [5, 6], an optimized protein extraction protocol [7], as well as a commercial kit for protein extraction from soils on full-scale activated sludge.

Activated sludge was taken from the aeration tank of the nutrient removal wastewater treatment plant at Aalborg West, Denmark [2], in November 2013. For each approach replicate 2 mL samples of activated sludge (4 g/L initial suspended solids) were harvested by centrifugation at 4500 *g* for 15 min and 4°C.

Cell lysis was achieved by either ultra-sonication, freeze, and thaw or bead beating. Ultra-sonication was performed on ice 3 times 30 s at an amplitude of 20%. Freeze and thaw was done in three cycles by freezing in liquid nitrogen and thawing in a ~45°C water bath. Bead beating used 4 cycles of 40 s at 6 m/s with 2 min breaks on ice in between. The remaining steps (see Table 1) were either a combination

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**Abbreviations:** **A**, alkaline and phenol treatment/ammonium acetate precipitation; **B**, B-PER detergent based protein extraction; **C**, thermal and SDS treatment / TCA precipitation; **COG**, cluster of orthologous groups; **K**, kit for protein extraction from soil

 

**Table 1.** Overview of the means (Ø) as well as standard deviations (σ) for the isoelectric point (p*I*), molecular weight (MW), number of MS/MS, and identified peptides (PepIDs) found for each extraction method

| Method | Ø p*I* | σ p*I* | Ø MW | σ MW | Ø MS/MS | σ MS/MS | Ø PepIDs | σ PepIDs |
|---|---|---|---|---|---|---|---|---|
| A | 6.58 | 1.56 | 46522 | 32767 | 36999 | 2059 | 1971 | 267 |
| B | 6.68 | 1.94 | 42415 | 29558 | 38320 | 749 | 2555 | 558 |
| C | 6.87 | 1.71 | 47599 | 33270 | 33184 | 6474 | 1688 | 586 |
| K | 6.81 | 1.82 | 41625 | 30989 | 43631 | 2906 | 2850 | 219 |

A, alkaline and phenol treatment / ammonium acetate precipitation, Kuhn et al. [5]; B, B-PER detergent based protein extraction, Barr et al. [7] (Supporting Information 1 and 2); C, thermal and SDS treatment / TCA precipitation, Chourey et al. [6]; K, kit for protein extraction from soil (MoBio).

of alkaline and phenol treatment followed by a protein precipitation in ammonium acetate in methanol proposed by Kuhn et al. (method A) [5], or an optimized protocol based on the protein extraction reagent (B-Per, Pierce) proposed by Barr et al. at the IWA Biofilm Conference 2011 (method B) [7] (see Supporting Information 1 and 2). Additionally, a protocol optimized for soils, applying thermally assisted detergent-based cellular lysis (SDS) followed by TCA precipitation as proposed by Chourey et al. (method C) [6], and a commercial kit for protein extraction from soils, were tested (method K, NoviPure® Soil Protein Extraction Kit, Mo-Bio). The commercial kit applies physical lysis similar, but presumably weaker (strong vortexing), to bead beating. Right before the final protein precipitation step, protein concentrations were measured by a modified Lowry protocol [8]. After protein precipitation the protein pellets were solubilized in Laemmli-buffer prior to 1D SDS-PAGE for removal of interfering substances. Short gels of less than 1 cm were subjected wholly to in-gel tryptic digestion [9]. Peptides were purified and desalted by $C_{18}$ microcolumns [10] and were then reconstituted in 20 μL 0.1% trifluoroacetic acid/2% acetonitrile. Of each sample 8 μL were injected by the autosampler and concentrated on a trapping column (Pepmap100, $C_{18}$, 100 μm × 2 cm, 5 μm, Thermo Fisher Scientific) with water containing 0.1% formic acid and 2% ACN at flow rates of 4 μL/min. After 10 min, the peptides were eluted into a separation column (PepmapRSLC, $C_{18}$, 75 μm × 50 cm, 2 μm, Thermo Fisher Scientific). Chromatography was performed with 0.1% formic acid in solvent A (100% water) and B (100% acetonitrile). The solvent B gradient was set in the first 4 min from 2 to 10% and subsequently increased to 30% in the next 125. After this solvent B was increased from 30% to 40% within 10 min with a final switch to 85% solvent B for an additional 10 min using a nano-high pressure liquid chromatography system (Ultimate3000 UHPLC, Thermo Fisher Scientific). Ionized peptides were measured and fragmented by a Q-Exactive mass spectrometer (Thermo Fisher Scientific). For an unbiased analysis continuous scanning of eluted peptide ions was carried out between 400 and 1200 $m/z$, automatically switching to MS/MS higher energy collisional dissociation mode and 12 MS/MS events per survey scan. For MS/MS higher energy collisional dissociation measurements a dynamic precursor exclusion of 30 s, peptide match and an apex trigger of 2–10 s, were enabled. The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRoteomics IDEntifications (PRIDE) partner repository [11] with the dataset identifier PXD000862.

To decrease the false-negative rate and to equalize the NCBInr and metagenome databases in size, a 2-step approach was used [12]. First, an artificial metagenome was created using weakly filtered protein identifications of a survey search. Raw MS and MS/MS data were processed by Thermo Proteome Discoverer software (v.1.4.0.288), Mascot (v. 2.3) and either the NCBInr database (v. December 17th, 2013) with a restriction to prokaryotic entries or a combination of two previously published metagenomes from the same [2] or a very similar site [1]. Oxidation of methionine was defined as variable modification and carbamidomethylation of cysteine as fixed modification. Precursor ion tolerance was set to 20 ppm and fragment ion tolerance to 0.05 Da. The relatively high tolerances were chosen to not restrict the final search space too much. Furthermore, all peaks besides the top 12 peaks per 100 Da in each MS/MS were removed to de-noise spectra before identification. Using Thermo´s Proteome Discoverer a protein fasta database was exported. Protein grouping was activated with a minimum peptide confidence of low and a delta Cn of 0.5. Strict maximum parsimony principle was deactivated. The exported protein fasta was then used as the database for the 2nd search. The final databases contained 26 868 and 43 908 entries for the metagenome and NCBInr derived fasta files, respectively. Maxquant (v. 1.4.1.2) [13] was applied and standard settings were kept. These included a peptide and protein FDR below 1%, at least 1 peptide [14], a precursor mass tolerance of 4.5 ppm after mass recalibration and a fragment ion mass tolerance of 20 ppm.

To evaluate the performance of the three different extraction methods and the kit, three technical replicates per extraction approach were performed. Evaluation of performance was done by measuring protein concentration of extracts, number of obtainable MS/MS spectra, number of possible peptide and protein identifications, overlap of peptide identifications, reproducibility for protein quantification and defining the bias toward specific phylogenetic groups as well as p*I* and protein mass.

As a first step, every extraction protocol was tested in combination with different lysis methods and the protein

 

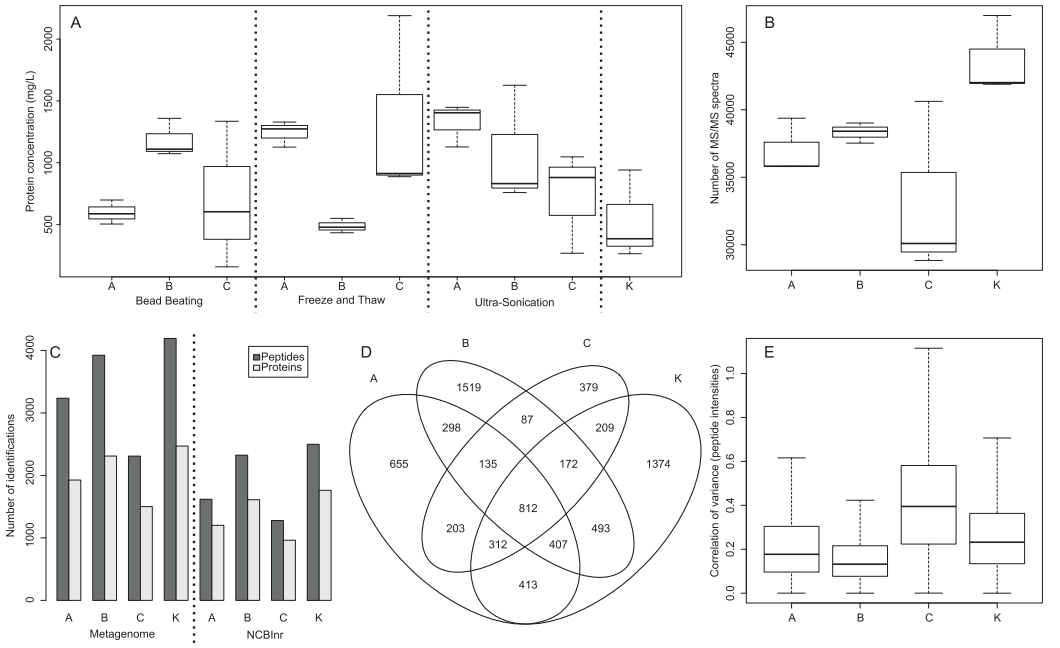


**Figure 1.** For a short explanation of the method abbreviations, A, B, C, and K see Table 1. For a *p*-value matrix (two-tailed, heteroscedastic *t*-test) of the boxplots A, B, and E, see Supporting Information 3. Outliers are not shown in the boxplots. (A) Measured protein concentrations after combining previously described protein extraction protocols with different lysis methods. Initial total suspended solids was approx. 4000 mg/L. (B) Boxplot of obtained MS/MS. (C) Number of identified peptides and proteins (FDR 1%, identified in two of three replicates) using 2-step databases based on metagenome sequences and NCBInr. (D) Overlap of identified peptides based on the metagenome after different extraction protocols. (E) Correlation of variance of median normalized peptide intensities from peptides identified against the metagenomes. 225 × 138 mm (300 × 300 DPI).

concentrations obtained were assessed (Fig. 1A). Interestingly, neither bead beating, freeze, and thaw nor ultrasonication were generally superior in terms of retrieved protein concentrations. In general, the tested extraction methods were already published in combination with the most efficient lysis method. Overall, method A in combination with freeze and thaw or ultra-sonication, as well as method B in combination with bead beating, showed highest protein concentrations and low variance. Method C showed a very high variance, which could be due to residual SDS. Furthermore, a high amount of humics was coextracted (data not shown). The commercial kit (K) for soil protein extraction showed a comparatively low protein concentration.

Samples from the best performing whole extraction methods and the kit were further analyzed by MS: A (ultrasonication), B (bead beating), C (freeze and thaw), and K (kit). In contradiction to their low protein concentrations, mass spectrometric analysis of the K samples showed the highest number of MS/MS spectra (Table 1, Fig. 1B) with a clear gap to samples from B and A. The reason for the discrepancy between protein concentration and MS/MS spectra for method K is unknown, but one explanation might be that it extracts relatively unbiased thus leading to an even distribution of extracted proteins and finally fragmentable peptides. Likewise to its high variance in protein concentration, method C resulted in a high variance of obtained MS/MS spectra. The positive identification rate averaged roughly 10% of the MS/MS for all samples.

One problem in metaproteomics is often the lack of appropriate sequence information. In these cases public databases like NCBInr (http://www.ncbi.nlm.nih.gov/) must be used. The size and unspecific nature of these databases usually leads to a high number of false negatives and makes comparison of the results with smaller and specific metagenomes unfeasible. One approach is the manual selection of organisms that are deemed to be present or important, but this can be heavily biased by personal assumptions. In this study, a 2-step database approach was used to equalize the search space of NCBInr and the used metagenome. Nevertheless, the use of specific sequence information clearly boosted the number of peptide and protein identifications possible for activated sludge (Fig. 1C). The performance in terms of identification

 

numbers for each extraction method was proportional to the number of MS/MS spectra (Fig. 1B and C). The kit (method K) showed the highest number of identifications with up to ~2300 proteins identified in at least two of three replicates, followed by methods B and A. Although this is the highest reported number of protein identification from activated sludge, it still only represents the protein identifications from roughly two species in pure cultures [15] and previous tests showed that low-diversity lab-scale reactors enable an increase in protein identifications by up to 100% (data not shown). This reflects the difficulty to obtain comprehensive metaproteomic extractions and identifications from activated sludge from full-scale plants, with hundreds of species present. Even with the best available procedure only the most abundant proteins will be detected due to instrumental limitations. However, although only a small fraction of the potential proteome is observable at the moment, metaproteomics is the only omics technique that proves transcription and translation and allows comprehensive quantification of proteins.

Although some extraction methods led to a higher number of peptide identifications, each method had a high number of unique peptide identifications (Fig. 1D). Depending on the aim of future studies, it might be feasible to combine different extraction methods to gain better coverage. The data obtained suggests that none of the methods biased strongly toward a specific protein mass or p*I* range (see Table 1). Functional classification by clusters of orthologous groups (COG) [16] was performed on the WebMGA [17] server with an *e*-value cutoff of $10^{-3}$ and the best hit was kept. The functional distribution was relatively equal (see Table 2), although method A seemed to bias against proteins related to translation. To assess if e.g. membrane or cytosolic proteins were favored by one or the other method, all proteins were blasted against PSORTdb [18] with an *e*-value cutoff of $10^{-3}$ and the best hit was kept. In general the distribution was similar to the one that can be expected from a pure culture experiment (see Table 2). Although the assignment between the methods was very similar, method B might favor cytosolic proteins. This could be connected to a more rigorous cell lysis by bead beating. In agreement with this, phylogenetic analysis of the peptides identified (based on NCBInr) using Unipept [11] indicated that method B showed a significant higher portion of peptides assigned to the Actinobacteria or Firmicutes (Table 2). Both phyla are Gram-positive, which are known for their robust cell walls [19]. It can be reasoned that these bacteria are only sufficiently lysed by rigorous treatment and are thus easily underrepresented, as was also the case in the previous metagenomic analysis of Aalborg East wastewater treatment plant [1]. Another, rather obvious but easily neglected limitation is that phylogenetic association of proteomic findings will always be restricted to known phyla if public available databases are used. Proteins from poorly characterized phyla like *Saccharibacteria* (former candidate phylum TM7) [1] can hardly be identified by proteomics and conserved peptides will easily be assigned to other, perhaps nonrelated organisms that prohibits exact phylogenetic analysis. Therefore, protein

**Table 2.** Percentages of identified peptides that could be mapped to specific phyla by Unipept [16] are shown. Ambiguous peptides were removed. Reference percentages (R) reflect the previously reported community composition of Aalborg West at another time point [1]. They are based on quantitative fluorescence in situ hybridization (FISH) and thus should not be influenced by extraction biases. The functional distribution of identified proteins (two of three replicates) is also shown as well as the distribution of the cellular localization of identified proteins (two of three replicates)

| | A (%) | B (%) | C (%) | K (%) | R (%) |
|---|---|---|---|---|---|
| **Phylum** | | | | | |
| Proteobacteria | 74.0 | 53.8 | 70.4 | 79.1 | > 20 |
| Nitrospirae | 8.9 | 9.8 | 16.2 | 6.6 | ~ 2 |
| Actinobacteria | 5.0 | 16.1 | 1.9 | 3.9 | ~ 21 |
| Firmicutes | 3.4 | 8.7 | 3.3 | 3.5 | ~ 4 |
| Chloroflexi | 3.4 | 5.3 | 2.0 | 2.0 | ~ 10 |
| Bacteroidetes | 1.7 | 1.8 | 3.1 | 1.9 | ~ 6 |
| Saccharibacteria | | | | | ~ 5 |
| Other phylae | 3.5 | 4.4 | 3.2 | 3.0 | NT |
| **COG** | | | | | |
| a | 0.1 | 0.0 | 0.0 | 0.0 | |
| c | 22.7 | 21.7 | 24.2 | 22.5 | |
| d | 0.2 | 0.0 | 0.4 | 0.1 | |
| e | 6.8 | 6.8 | 4.9 | 6.6 | |
| f | 0.6 | 0.7 | 1.3 | 1.0 | |
| g | 10.2 | 12.7 | 7.6 | 8.5 | |
| h | 1.3 | 2.0 | 0.9 | 1.4 | |
| i | 5.2 | 5.8 | 4.9 | 4.7 | |
| j | 9.9 | 19.4 | 16.5 | 17.2 | |
| k | 3.0 | 2.8 | 5.2 | 3.7 | |
| l | 1.3 | 1.1 | 1.8 | 1.3 | |
| m | 4.8 | 3.8 | 4.4 | 4.0 | |
| n | 0.3 | 0.1 | 0.1 | 0.5 | |
| o | 6.6 | 4.0 | 5.9 | 5.3 | |
| p | 2.2 | 1.8 | 1.9 | 2.8 | |
| q | 1.0 | 0.6 | 0.7 | 1.0 | |
| r | 3.4 | 3.4 | 2.5 | 3.7 | |
| s | 2.2 | 1.8 | 2.5 | 2.6 | |
| t | 0.8 | 0.9 | 1.1 | 1.0 | |
| u | 1.3 | 0.2 | 0.1 | 0.3 | |
| v | 0.2 | 0.1 | 0.4 | 0.2 | |
| z | 0.1 | 0.0 | 0.1 | 0.1 | |
| NA | 8.8 | 5.6 | 8.0 | 6.8 | |
| Ambiguous | 6.7 | 4.6 | 4.6 | 4.9 | |
| **Localization** | | | | | |
| Cytoplasmic | 56.1 | 68.3 | 64.6 | 62.2 | |
| Cytoplasmic membrane | 9.8 | 7.1 | 10.2 | 7.6 | |
| Periplasmic | 10.0 | 5.8 | 6.2 | 9.3 | |
| Outer membrane | 6.7 | 3.6 | 4.2 | 4.1 | |
| Cellwall | 0.4 | 0.6 | 0.2 | 0.3 | |
| Extracellular | 1.2 | 0.7 | 0.9 | 1.1 | |
| Unknown | 15.0 | 13.3 | 13.3 | 14.8 | |
| NA | 0.7 | 0.6 | 0.4 | 0.6 | |

(a) RNA processing and modification, (c) energy production and conversion, (d) cell-cycle control and mitosis, (e) amino acid metabolism and transport, (f) nucleotide metabolism and transport, (g) carbohydrate metabolism and transport, (h) coenzyme metabolism, (i) lipid metabolism, (j) translation, (k) transcription, (l) replication and repair, (m) cell wall/membrane/envelop biogenesis, (n) cell motility, (o) PTM, protein turnover, chaperone functions, (p) inorganic ion transport and metabolism, (q) secondary structure, (r) general functional prediction only, (s) function unknown, (t) signal transduction, (u) intracellular trafficking and secretion, (v) defense mechanisms, (z) cytoskeleton; (Ambiguous) several categories; (NA) no hit above threshold, (NT) not determined.
All of these results are based on identifications from the NCBInr-based search.

 

identifications will be skewed towards well-investigated phyla like *Proteobacteria*.

Besides providing a correct reflection of community composition and high proteome coverage, a protein extraction method should also deliver reproducible data for protein quantification. To assess this, the correlation of variance of the median normalized peptide intensities was calculated (Fig. 1E). The best reproducibility could be achieved by method B, followed by A and K.

Although the extraction protocol based on bead beating and employment of B-Per did not show the highest number of identifications, it gave improved access to Gram-positive organisms and showed a high reproducibility in terms of peptide intensities for protein quantification within full-scale activated sludge. The optimization of extraction methods will enable further proteome studies of activated sludge in situ. Furthermore, use of specific metagenomes and the application of 2-step database approaches improved the identification of peptides and proteins.

*The MS proteomics data in this paper have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [11]: dataset identifier PXD000862.*

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*The authors have declared no conflict of interest.*

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# CHAPTER 4. DIRECT IDENTIFICATION OF FUNCTIONAL AMYLOID PROTEINS BY LABEL-FREE QUANTITATIVE MASS SPECTROMETRY

**Paper 3:**

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* These authors have contributed equally to this work.

biomolecules

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Article

# Direct Identification of Functional Amyloid Proteins by Label-Free Quantitative Mass Spectrometry

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**Abstract:** Functional amyloids are important structural and functional components of many biofilms, yet our knowledge of these fascinating polymers is limited to a few examples for which the native amyloids have been isolated in pure form. Isolation of the functional amyloids from other cell components represents a major bottleneck in the search for new functional amyloid systems. Here we present a label-free quantitative mass spectrometry method that allows identification of amyloid proteins directly in cell lysates. The method takes advantage of the extreme structural stability and polymeric nature of functional amyloids and the ability of concentrated formic acid to depolymerize the amyloids. An automated data processing pipeline that provides a short list of amyloid protein candidates was developed based on an amyloid-specific sigmoidal abundance signature in samples treated with increasing concentrations of formic acid. The method was evaluated using the *Escherichia coli* curli and the *Pseudomonas* Fap system. It confidently identified the major amyloid subunit for both systems, as well as the minor subunit for the curli system. A few non-amyloid proteins also displayed the sigmoidal abundance signature. However, only one of these contained a sec-dependent signal peptide, which characterizes most of all secreted proteins, including all currently known functional bacterial amyloids.

**Keywords:** functional amyloids; biofilm; nanomaterials; mass spectrometry

## 1. Introduction

Amyloids are highly ordered protein fibrils defined by a cross-β-sheet quaternary structure and the ability to self-assemble from their monomeric counterparts in a nucleation-dependent process [1]. Many amyloids display exceptional resistance towards thermal and chemical denaturants due to a tightly packed cross-β structure [2]. Consequently, they are ideal structural materials in biological systems, and organisms from all domains of life exploit amyloids for beneficial purposes [3,4]. Functional amyloids have also shown promising results as tunable nanomaterials. The well-described *Escherichia coli* curli system was for example used to engineer biofilm properties by genetically attaching functional domains from other proteins to the major amyloid subunit (CsgA) [5]. Another type of amyloid that has shown potential applications as a nanomaterial is the hydrophobins expressed by fungi. Hydrophobins have a special ability to position themselves at water-air or solid-water interfaces, thus making them very applicable within protein purification. Furthermore, they show possibilities within the food industry, in pharmaceuticals, and for biotechnological processes [6]. With the numerous

possible applications of amyloids, it is of great interest to discover new amyloid systems from a variety of different microorganisms.

Microbial biofilms represent bacteria embedded in an extracellular matrix (ECM) composed mainly of polysaccharides, extracellular DNA, and proteins [7]. The ECM enables the bacteria to organize spatially, which allows for functional differentiation [8,9]. It also provides protection against environmental and chemical stresses, including the action of antibiotics and host immune response. The biofilm lifestyle is accordingly favored by the majority of all bacteria [9], and biofilm formation by pathogenic bacteria is tightly associated with the development of chronic infections [10]. Previous studies have indicated that amyloids are common components of almost all microbial biofilms independent of habitat [11,12]. They are therefore believed to play essential roles in biofilm ecology [13]. However, our current knowledge of functional amyloids is restricted to a few amyloid systems, for which the amyloids have been purified and characterized in detail [4]. The identification and characterization of more amyloid systems will likely provide a deeper insight into the many roles of amyloids in biofilms.

Functional amyloid systems are traditionally discovered based on the isolation of native amyloids and the validation of the amyloid structure through biophysical characterization. The isolation of amyloids is unfortunately not a straightforward task. Insolubility and extreme stability, which characterize most functional amyloids, exclude them from standard protein analyses, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry-based characterization without special pretreatment. Furthermore, many functional amyloids are highly adhesive and therefore easily lost during purification due to binding to consumables such as pipet tips and sample tubes. Consequently, the current methods require pure cultures, which can express a large number of amyloids under laboratory conditions. A purification-independent technique for amyloid protein identification would provide the means to identify less abundant amyloids, as well as amyloids that are only expressed upon exposure to habitat-specific environmental cues, including stress conditions and host defense mechanisms [14].

We here present a quantitative proteomics technique that facilitates the direct identification of functional amyloid candidates in cell lysates. This technique is specific, sensitive, and provides an opportunity to identify amyloids in complex samples with low bacterial diversity, such as clinical biofilms.

## 2. Results

### *2.1. Description of the Method*

An overview of the new method for amyloid detection is presented in Figure 1. The bacteria in the sample are first lysed to release intracellular proteins. Next, aliquots of the total cell lysate are lyophilized and treated with formic acid at concentrations ranging from 0% to 100%. Many functional amyloids are only depolymerized in aggressive solvents such as concentrated formic acid [15,16], trifluoroacetic acid (TFA) [17,18], or hexafluoroisopropanol (HFIP) [19]. Functional amyloids are therefore only depolymerized in the samples treated with high concentrations of formic acid. The samples are lyophilized again to remove water and formic acid, dissolved in a special reducing SDS-PAGE loading buffer [20], and subjected to a brief run on an SDS-PAGE gel. The SDS-PAGE has two purposes: it removes cell debris and the native amyloids that are not able to enter the gel, and it retains the depolymerized amyloid proteins in an exposed monomeric conformation amenable to proteolytic digestion. The gel-embedded proteins are subjected to in-gel tryptic digest, and the resulting peptides are analyzed by mass spectrometry. Protein identification and quantification are done using MaxQuant [21] with the label-free quantification (LFQ) algorithm [22]. The LFQ values are normalized between individual measurements to prevent systematic errors such as deviations in sample loading, and are based on at least two quantifiable peptides. This allows the relative abundances of the same protein to be compared across samples. Amyloid proteins are suspected to

show much higher abundance in samples treated with a higher concentration of formic acid, and they will therefore produce a characteristic sigmoidal abundance signature when the relative abundance is plotted against the formic acid concentration (Figure 1).

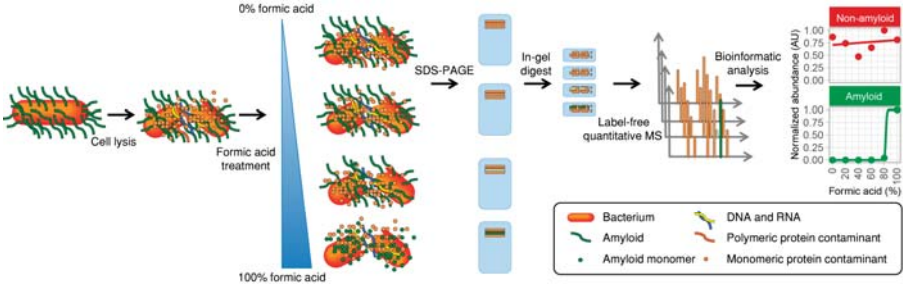


**Figure 1.** Direct identification of functional amyloid proteins using label-free quantitative (LFQ) Liquid chromatography–tandem mass spectrometry (LC-MS/MS). The sample is lysed and divided into aliquots that are lyophilized and treated with either 0%, 20%, 40%, 60%, 80%, or 100% formic acid. The samples are then lyophilized, dissolved in reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and subjected to short run SDS-PAGE. The amyloid proteins can only enter the gel if they have been pretreated with concentrated formic acid and are therefore only present in these samples. In-gel digestion is carried out with trypsin, and samples analyzed by label-free quantitative LC-MS/MS using MaxQuant and the MaxLFQ algorithm. The data is finally analyzed for each protein using an automated script, and positive amyloid candidates are identified based on their abundance profiles with respect to the formic acid concentration.

The mass spectrometry analysis results in the identification of thousands of proteins, and it is an enormous task to review the abundance signature for each protein. To ease this process, we created an R-markdown script that automatically identifies functional amyloid candidates based on their abundance signatures (Script S1). The script uses the raw MaxQuant data as input and separates the data for each protein. It then normalizes the abundance data according to the sample with the highest concentration giving it a value of 1. The normalized data for each protein is then fitted to a generalized linear model of the logistic function:

$$f(x) = 1/(1 + \exp(-(a + bx))), \quad (1)$$

The formic acid concentration where half of the amyloids have been depolymerized (f50) is:

$$f50 = -a/b, \quad (2)$$

The slope of the fit at f50 is calculated as:

$$f'(f50) = b/4, \quad (3)$$

We here define functional amyloids candidates as proteins that fulfill the following requirements:

$$\begin{aligned} f50 &> 60, \\ f50 &< 100, \\ f'(f50) &> 0.025 \end{aligned} \quad (4)$$

### 2.2. Method Validation with Cell Lysates of E. coli SM2258

The method was first evaluated with four biological replicates of the amyloid-producing *E. coli* strain SM2258 [11] grown at conditions known to promote curli expression (Figure 2A). The curli amyloids are composed of two subunits, the major subunit CsgA and the minor subunit CsgB [23].

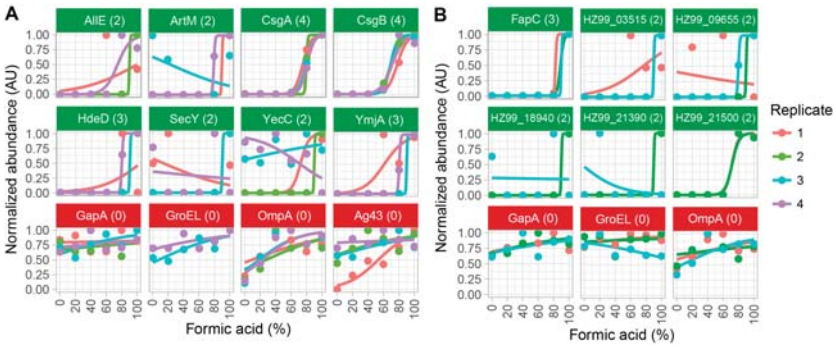


**Figure 2.** The identification of functional amyloid candidates in cell lysates of (**A**) *Escherichia coli* SM2258 and (**B**) *Pseudomonas* sp. UK4. Proteins with amyloid-specific abundance signatures in at least two biological replicates are shown with green titles. Negative controls are shown in red titles, which include the household proteins, Glyceraldehyde-3-phosphate dehydrogenase A (GapA) and 60 kDa chaperonin (GroEL); and β-barrel outer membrane proteins, like outer membrane protein A (OmpA) and antigen 43 (Ag43). The numbers in the parentheses indicate the number of biological replicates in which the proteins were classified as amyloid candidates based on the fitting parameters. Notice that some data points and curves are hidden as they overlap, and some proteins were not observed in all replicates.

Mass spectrometry analysis identified a total of 1438 proteins (Figure S1), of which 56 were classified as functional amyloid candidates (Table S1). However, many of the identified candidates were low abundant proteins that were only supported by data from a single biological replicate. To remove false positive hits, only proteins that were identified as amyloids in at least two of the replicates were considered. This reduced the list of amyloid candidates to eight (Figure 2). All currently known functional bacterial amyloids contain Sec-dependent signal peptides that mark them for secretion. The identified candidates were therefore analyzed for the presence of sec-dependent signal peptides using the SignalP 4.1 algorithm [24] (Table S1). Only two of the eight amyloid candidates contained Sec-dependent signal peptides, namely the major (CsgA) and the minor (CsgB) curli amyloid subunits. These two proteins were also the only proteins identified as amyloid candidates in all four biological replicates, demonstrating the specificity and sensitivity of the method.

### 2.3. Method Validation with Cell Lysates of Pseudomonas sp. UK4

To further evaluate the method, we analyzed three biological replicates of *Pseudomonas* sp. UK4 grown at conditions known to promote functional amyloids of *Pseudomonas* (Fap) expression [16] (Figure 2B). The Fap amyloids are composed of the major subunit FapC and the minor subunit FapB [16]. A third protein FapE has also been observed in the purified preparations of Fap fibrils, but it is uncertain if this protein is an integrated part of the amyloid [20]. *Pseudomonas* sp. UK4 produce significantly less amyloid compared to *E. coli* SM2258, and is likely a better model for other wild-type strains producing amyloids.

Mass spectrometry analysis identified a total of 2309 proteins (Figure S2), of which 46 were classified as functional amyloid candidates (Table S2). Only six of these were supported by data from at least two replicates. Two of these proteins contained Sec-dependent signal peptides according

to SignalP 4.1 analysis. One was the major functional amyloid subunit (FapC), the other was a low abundant glutamine ABC transporter substrate-binding protein (HZ99_18940). Only FapC was identified in all three biological replicates. The minor amyloid subunit (FapB) was not detected in the mass spectrometry data due to low abundance. The confident detection of FapC as an amyloid protein, even though it was expressed at a significantly lower level than the *E. coli* curli, confirms the specificity and sensitivity of the method.

## 3. Discussion

The isolation of functional amyloids from other cell components represents a major obstacle in the identification of new functional amyloid systems [4]. The method described here allows identification of functional amyloid proteins directly in cell lysates without any purification steps. It thus provides an opportunity to screen for amyloids in systems, where it is difficult or impossible to separate the native amyloids from contaminating cell components. By applying the method to two evolutionarily distinct amyloid systems, it was shown that the method is both sensitive and specific. The method returned only a few false positive amyloid candidates when biological replicates were considered, and the number of false positives could easily be reduced based on the presence or absence of Sec-dependent signal peptides, which characterizes all currently described functional bacterial amyloid proteins. However, cell lysis products are known to play important roles in biofilm structure and function, and it is possible the cytosolic proteins released may form amyloids when exposed to the extracellular environment. Such amyloids would be missed with the signal peptide criterion.

Another problem with the identification of novel functional amyloid systems relies on the fact that many bacteria require specific environmental stimuli to activate amyloid expression [25]. Such stimuli may involve interaction with host surface molecules, or they may be too complex to replicate in the laboratory. However, the current method may provide a solution to this problem. Due to the high sensitivity of the method, it may allow for the identification of amyloids directly in complex samples such as environmental biofilms or in cocultures of bacteria and their hosts.

We have previously hypothesized that many biofilm-forming pathogenic bacteria may use amyloid proteins during infections [26,27]. Now we can test this hypothesis by applying the current method to samples obtained from chronic wounds of immune-impaired individuals or sputum samples from patients suffering from cystic fibrosis. If amyloids are identified as important virulence factors, they may be targeted with specific or more general inhibitors, represented by CsgC for *E. coli* [28] and epigallocatechin gallate (EGCG) for *Pseudomonas* Fap [29], respectively.

It should be stressed that the method itself cannot be used to confirm whether a protein is an amyloid component. This still requires biophysical characterization of the native amyloid fibrils. However, with the current method and access to genomes or the relevant strains, we can identify the operons encoding the amyloids. These operons may then be expressed in bacteria that allow purification of the native amyloids, as has previously been done for the *fap*-operon from various species of *Pseudomonas* [16,20]. Another limitation of the method is that it can only be used to identify extremely stable amyloids that require more 50% formic acid to be depolymerized. The TasA amyloids from *Bacillus subtilis* are known to be depolymerized already at 20% formic acid [30]. The method will therefore not identify TasA as an amyloid protein. The current approach also misses proteins that occur both in a soluble and an amyloid form within the same sample, e.g., the phenol soluble modulins of *Staphylococcus aureus*. However, despite these limitations, we believe that the current method will provide a useful tool for many working within the field of functional amyloids, and that it will expedite the identification of future functional amyloid systems.

## 4. Materials and Methods

### *4.1. Bacterial Cultures and Activated Sludge*

Glycerol stocks of *E. coli* MG1665 str. SM2258 [11] and *Pseudomonas* sp. UK4 [16,31] were used to inoculate 10 mL of colonization factor antigen (CFA) medium (10 g/L hydrolyzed casein, 50 mg/L MgSO4, 5 mg/L MnCl2, 1.5 g/L yeast extract, pH 7.4) in 50-mL Greiner tubes. The bacteria were grown overnight at 26 °C with 150 rpm of agitation in an Innova 40 Benchtop Incubator Shaker (Eppendorf, Hamburg, Germany). Then, 100 µL aliquots of each overnight culture were spread on 10 CFA agar plates solidified with 2% agar, which were incubated at 26 °C for 72 h. Bacteria from 10 plates were scraped off and resuspended in 10 mL buffer (10 mM Tris-HCl, pH 8.0).

### *4.2. Sample Preparation for Mass Spectrometry*

For sample preparation, 4 × 1 mL of bacterial culture were transferred to lysing matrix E tubes (MP Biomedicals, Eschwege, Germany). Subsequently, 10 µL of Halt protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA, USA) were added to each tube to prevent protein degradation during cell lysis. The samples were then lysed by bead beating in a FastPrep-24 instrument for 3 × 20 s at 6.0 m/s. The samples were incubated on ice for 2 min between each bead beating to prevent thermally induced protein denaturation. Beads were allowed to settle by gravity for 5 min, after which 500 µL cell lysate were collected from the four tubes and combined. Then, 50 µL aliquots of the cell lysate were transferred to six Eppendorf tubes in three or four replicates. The replicates were lyophilized and resuspended in 100 µL of either 0%, 20%, 40%, 60%, 80% or 100% formic acid. The samples were lyophilized again and then resuspended in 100 µL of reducing SDS-PAGE loading buffer containing 8 M of urea [20]. Insoluble material was pelleted by centrifugation for 1 min at 22,000× *g*, and 15 µL of supernatant was loaded on 12% SDS-PAGE gels. Electrophoresis was carried out at 140 V for 5 min, and the gels were stained with Coomassie Brilliant Blue G250 (ThermoFisher Scientific). The narrow bands containing all proteins were excised and subjected to tryptic in-gel digestion [32]. Tryptic peptides were reconstituted in 5% formic acid and purified using StageTips packed with PorosOligo R3 material (Applied Biosystems, Foster City, CA, USA) on top of two C18 disks (3 M, Bioanalytical Technologies, St. Paul, MN. USA) as previously described [33,34]. Peptides were eluted with 66% (*v*/*v*) acetonitrile (ACN) and dried by vacuum centrifugation without heating.

### *4.3. Mass Spectrometry*

Ultra-performance liquid chromatography (UPLC) tandem mass spectrometry analysis was performed on an ultimate 300 UPLC system (ThermoFisher Scientific) coupled online to a Q Exactive Plus mass spectrometer (ThermoFisher Scientific). Desalted peptides were reconstituted in 0.1% trifluoroacetic acid and 2% acetonitrile. Of each sample, 8 µL were injected by the autosampler and concentrated on a trapping column (Pepmap100, C18, 100 µm × 2 cm, 5 µm, ThermoFisher Scientific) with water containing 0.1% formic acid and 2% ACN at flow rates of 4 µL $min^{-1}$. After 5 min, the peptides were loaded onto a separation column (PepmapRSLC, C18, 75 µm i.d. × 75 cm, 100 Å, ThermoFisher Scientific). Chromatography was performed with 0.1% formic acid in solvent A (100% water) and B (100% acetonitrile) using a ramp gradient. The concentration of B was increased from 2% to 8% over 1 min, followed by an increase from 8% to 30% over 39 min. Solvent B was subsequently increased from 30% to 90% within 5 min and maintained at this level for 3 min.

The mass spectrometry proteomics data was deposited to the ProteomeXchange Consortium via the PRIDE [35] partner repository with the dataset identifier PXD006835.

### *4.4. Data Analysis*

Protein identification and quantification were done with the open-source software MaxQuant v1.5.8.3 [21]. The sequence databases for *E. coli* str. K-12 MG1655 and *Pseudomonas* sp. UK4 were retrieved from the National Center for Biotechnology Information (NCBI) (tax: 511145 and 452680) [36].

Besides the standard settings, LFQ [22] was activated in MaxQuant. This included a peptide and protein false discovery rate of 1%. Reversed sequences as decoys and contaminant sequences were added automatically by MaxQuant. The minimum ratio count for LFQ was set to one. Complete lists of the protein and peptide identifications, as well as detailed parameters, are available together with the deposited mass spectrometric data. The reverse and contaminant sequences were removed from the MaxQuant output, and unique identifiers (gene names or id numbers) were created for each protein. The resulting dataset was loaded into R and analyzed using an automated R-markdown script (Script S1).

**Supplementary Materials:** The following are available online at www.mdpi.com/2218-273X/7/3/58/s1, Figure S1: Abundance profiles for all proteins in the *E. coli* SM2258 sample, Figure S2: Abundance profiles for all proteins in the *Pseudomonas* sp. UK4 sample, Table S1: Amyloid candidates identified in the *E. coli* SM2258 sample, Table S2: Amyloid candidates identified in the *Pseudomonas* sp. UK4 sample, Script S1: R-markdown script used for automated identification of amyloid protein candidates.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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# CHAPTER 5. FUNCTIONAL AMYLOIDS KEEP QUORUM-SENSING MOLECULES IN CHECK

**Paper 4:**

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# Functional Amyloids Keep Quorum-sensing Molecules in Check*

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**Background:** In biofilms, bacteria communicate via quorum-sensing (QS) molecules.
**Results:** The specific binding affinity of QS molecules to a functional amyloid is determined.
**Conclusion:** Functional amyloids can transiently bind and retain QS molecules.
**Significance:** Functional amyloids are important for cell signaling within biofilms.

**The mechanism by which extracellular metabolites, including redox mediators and quorum-sensing signaling molecules, traffic through the extracellular matrix of biofilms is poorly explored. We hypothesize that functional amyloids, abundant in natural biofilms and possessing hydrophobic domains, retain these metabolites. Using surface plasmon resonance, we demonstrate that the quorum-sensing (QS) molecules, 2-heptyl-3-hydroxy-4(1H)-quinolone and *N*-(3-oxododecanoyl)-L-homoserine lactone, and the redox mediator pyocyanin bind with transient affinity to functional amyloids from *Pseudomonas* (Fap). Their high hydrophobicity predisposes them to signal-amyloid interactions, but specific interactions also play a role. Transient interactions allow for rapid association and dissociation kinetics, which make the QS molecules bioavailable and at the same time secure within the extracellular matrix as a consequence of serial bindings. Retention of the QS molecules was confirmed using *Pseudomonas aeruginosa* PAO1-based 2-heptyl-3-hydroxy-4(1H)-quinolone and *N*-(3-oxododecanoyl)-L-homoserine lactone reporter assays, showing that Fap fibrils pretreated with the QS molecules activate the reporters even after sequential washes. Pyocyanin retention was validated by electrochemical analysis of pyocyanin-pretreated Fap fibrils subjected to the same washing process. Results suggest that QS molecule-amyloid interactions are probably important in the turbulent environments commonly encountered in natural habitats.**

Amyloids are insoluble fibers formed by either regulated or unregulated protein folding. Although they are more commonly associated with human diseases, amyloids fulfill important microbial functions, including as structural components in biofilms and in the cell wall of bacterial spores, adhesins mediating specific binding to and internalization into host cells, and as a means to attenuate the cytotoxicity of bacteriocins (1–4). Furthermore, their assembly is regulated (5), suggesting that such functions are not chance occurrences.

Bacterial amyloids have been detected in natural biofilms from a range of aquatic systems, including water reservoirs, seawater, and wastewater treatment-activated sludges (6, 7). Given their apparent abundance in natural biofilms and the tantalizing prospect of using them to better understand the formation of disease-related amyloids (4), there is a surprising paucity of information on the structure-function dependence of amyloids and the means by which this is impacted by interactions with other molecules. The curli fibers of *Escherichia coli* and *Salmonella enterica*, Fap fibrils of *Pseudomonas* spp., and TasA of *Bacillus subtilis* have each received attention (2, 8, 9). Their roles in biofilms have been described variously as those of adhesins, stabilizers (*e.g. B. subtilis*) (8), and promoters of the initial biofilm formation (9). They have also been suggested to be virulence determinants, as with the curli fimbriae of enterohemorrhagic, enterotoxigenic, and sepsis-derived strains of *E. coli* (10). However, with some notable exceptions (11), as with functional analyses of other extracellular polymeric substances (EPS),[3] work with amyloids has generally been observational rather than mechanistic.

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[3] The abbreviations used are: EPS, extracellular polymeric substance(s); ThT, thioflavin T; QS, quorum-sensing; 3-oxo-C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; PQS, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 4-hydroxy-2-heptylquinoline; SPR, surface plasmon resonance; FAP, functional amyloid(s) from *Pseudomonas*.

*Pseudomonas aeruginosa* is an opportunistic bacterial pathogen (12). Its virulence is associated with the ability to form biofilms (13), which are the dominant mode of microbial life in many engineered, medical, and natural settings (14). Furthermore, the extracellular domain of *P. aeruginosa* biofilms is well understood chemically in terms of the EPS composition and metabolites produced. Thus, *P. aeruginosa* is often used in biofilm studies, including those addressing the effects of its amyloids on biofilm formation. Dueholm *et al.* (15) discovered a six-gene operon encoding the expression of functional amyloids (*fapA–F*) in *P. aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. Overexpression of Fap fibrils, which are composed mainly of FapC, has since been shown to increase biofilm formation (9).

Bacteria collectively coordinate their behavior and communicate with each other using quorum-sensing (QS) systems, whereby small molecules are secreted and elicit changes in gene expression in those cells responding to them (16). Four QS systems have been identified for *P. aeruginosa*, including the Las and Rhl systems, which employ the autoinducers *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone, respectively (17, 18); the *cis*-2-decenoic acid signaling system (19); and the 4-quinolone signal system, unique to the members of the genus *Pseudomonas*, which involves 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) and the transcriptional regulator PqsR (20). PQS is a putative iron chelator, which positively regulates the Rhl QS system (21). The Rhl system in turn modifies elastase (LasB) and lectin (LecA) production. Furthermore, the PQS system utilizes the redox shuttle pyocyanin as its terminal signaling factor (16). Pyocyanin is a heterocyclic compound that belongs to the phenazine group, which when produced by *P. aeruginosa* enhances bacterial virulence (*e.g.* in chronic lung infections) and may also provide the bacterium with a competitive advantage as a result of its antimicrobial activity (22). QS molecules in *P. aeruginosa* are chemically distinct with different targets and a cascade of integrated and secondary effects that confound our understanding of their phenotypic consequences. However, they are all transmitted extracellularly, and they are all hydrophobic (23).

Although both the *P. aeruginosa* QS systems and its extracellular matrix have been studied extensively, the importance of possible interactions between the matrix components and QS molecules has not attracted the same level of interest. QS chemicals are transmitted through the extracellular domain. Mashburn and Whiteley (24) addressed the question of how transmission of hydrophobic QS molecules might be managed extracellularly by the packaging of a QS molecule into membrane vesicles before export. However, this was specific to PQS. Interactions between extracellular polymers and signaling chemicals are also probably crucial to their movement through biofilms. For example, it is believed that the immediate precursor of PQS, 4-hydroxy-2-heptylquinoline (HHQ), acts as a "messenger" molecule because it is secreted extracellularly and is then converted to PQS by other responding cells (21). Biofilms can form in flowing, often highly turbulent aquatic environments like rivers or wastewater treatment plants (25, 26), where the loss of small molecules by high diffusion rates is a significant challenge for bacteria. These habitats also mostly consist of complex, mixed microbial communities, raising interesting challenges around signal cross-talk and uptake by non-self organisms (14). Dilution or interception and potential interference or corruption of chemical signals represent a serious challenge to the bacteria. Although it is assumed that the EPS matrix allows biofilms to retain such molecules under non-quiescent conditions (27), the ability of a single EPS to facilitate this by a reversible binding process has never been demonstrated.

There are currently no high-resolution protein structures of biofilm-associated functional amyloids. However, amyloid fibers of the HET-s(218–289) prion and the $\beta$-amyloid peptide (A$\beta$(1–40)) associated with Alzheimer disease arise from $\beta$-solenoids that are stabilized by a hydrophobic triangular core (28, 29). The current structural model of the Fap fibrils suggests that their amyloids also contain similar hydrophobic domains (9). Such domains represent potential binding sites for hydrophobic signaling molecules. In the present study, amyloids were identified as an EPS component that may play a role in binding signaling metabolites. We investigated such a functional role by characterizing the binding affinities of QS molecules and other small hydrophobic and non-hydrophobic metabolites to amyloid polymers using surface plasmon resonance (SPR) biosensor technology. We present two important physiological consequences of this binding. Under non-quiescent conditions, amyloids can act as storage reservoirs to enhance information retention and electroconductivity, thus providing the first direct evidence for a specific role for an EPS component of a bacterial biofilm in signal molecule binding.

## EXPERIMENTAL PROCEDURES

*Strains—P. aeruginosa* PAO1 was obtained from the Genetic Stock Center (strain PAO0001). The PQS reporter strain was constructed by introducing the promoter-probe plasmid pAC37, containing a $P_{pqsA}$::*gfp(ASV)* transcriptional fusion, into *P. aeruginosa* PAO1 Δ*pqsC*, as described by Yang *et al.* (30). PQS positively regulates the *pqsABCDE* operon via PqsR (21). Thus, *pqsABCDE* expression indicates interaction of PQS with the cell (31). The *P. aeruginosa* PAO1 Δ*pqsC* mutant, which is unable to synthesize the PQS precursor HHQ, was obtained from a transposon mutagenesis library screen (31). The 3-oxo-C12-HSL reporter strain was obtained by introducing a $P_{lasB}$::*gfp(ASV)* transcriptional fusion and an extra copy of *lasR* under the influence of the *lac* promoter, which is constitutively expressed in *P. aeruginosa* into *P. aeruginosa* PAO1 Δ*lasI*Δ*rhlI* via a mini-Tn5 insert, as described by Yang *et al.* (32). 3-Oxo-C12-HSL is thus sensed through the LasR reporter, which in turn regulates the *lasB* promoter (32). Construction of the isogenic *P. aeruginosa* PAO1 Δ*lasI*Δ*rhlI* strain, which is unable to synthesize 3-oxo-C12-HSL, is described elsewhere (33).

*Metabolites*—PQS and HHQ were synthesized as described previously (21), and 25 mM stock solutions were prepared by dissolution in DMSO (25 mM). A stock solution of pyocyanin from *P. aeruginosa* (from Sigma-Aldrich) (480 $\mu$M) was prepared in DMSO.

*Expression and Isolation of Monomeric FapC*—A synthetic gene corresponding to the mature FapC protein from *P. aerugi-*

*nosa* PAO1 flanked by XbaI and XhoI restriction sites was designed using CLC Main Workbench version 6.0 and synthesized by GenScript (Germany). The synthetic gene was cloned into the pET32b(+) vector (Novagen) using the XbaI and XhoI restriction sites. This added a C-terminal His tag to the construct. The plasmid was transformed into *E. coli* BL21(DE3) (Invitrogen). Protein expression was carried out in 500 ml of LB medium (37 °C, 200 rpm). Induction was done with 1 mM isopropyl 1-thio-β-D-galactopyranoside at $A_{600\ nm} = 0.7–1.2$, and cells were harvested 3 h after induction by centrifugation (10,000 × *g*, 30 min). The cell pellet was resuspended in 25 ml of extraction buffer (6 M guanidinium chloride, 20 mM potassium phosphate, 500 mM NaCl, pH 7.4) and sonicated three times for 1 min each. The sample was placed on ice for 1 min between sonications. The lysate was then incubated overnight at 4 °C with gentle shaking. Insoluble material was pelleted by centrifugation (20,000 × *g*, 30 min). The His-tagged FapC proteins were isolated from the supernatant using a 5-ml HisTrap HP column (GE Healthcare) and an elution buffer corresponding to the extraction buffer containing 500 mM imidazole.

*Preparation of Natively Seeded FapC Fibrils*—Purified monomers in elution buffer were desalted using a PD-10 desalting column (GE Healthcare) equilibrated with deionized water. Protein concentration was estimated by UV absorbance of the peptide bond using the following extinction coefficient for the recombinant protein: $\epsilon_{280\ nm} = 0.701\ cm^{-1}\cdot(g/liter)^{-1}$. The protein was diluted in deionized water to 1 mg/ml and mixed with an equal amount of buffer (100 mM Tris-HCl, pH 7.4) supplemented with 50 μg/ml sonicated native Fap fibrils, which acted as a template to ensure the assembly of FapC fibrils with a native structure. The native Fap fibrils were purified from *P. aeruginosa* PAO1 overexpressing the *fap* operon as described by Dueholm *et al.* (9). 200-μl samples were loaded in a 96-well plate. Thioflavin T was added to a final concentration of 40 μM in a subset of the wells to visualize the fibrillation process. Immediately afterward, the plate was transferred to a Tecan GENios Pro plate reader, and ThT fluorescence was measured using excitation at 448 nm, emission at 485 nm, and a gain of 60. Measurements were obtained by bottom reads every 2 min, and 30 s of shaking (orbital, amplitude 2.5 mm) was applied between the reads. Reads were integrated for 40 μs. Three fibrillation curves were averaged to reduce the signal/noise ratio. The samples without ThT were collected, sonicated, and used to seed a new generation of recombinant FapC fibrils as described above. This step was repeated twice to ensure a uniform fibril batch without non-protein contaminants originating from the native fibrils.

*Expression, Isolation, and Fibrillation of α-Synuclein*—Recombinant α-synuclein was purified and fibrillated *in vitro* by shaking-induced fibrillation as described elsewhere (34).

*Transmission Electron Microscopy*—Amyloid fibrils were mounted on 400-mesh carbon-coated, glow-discharged nickel grids for 30 s. Grids were washed with one drop of double-distilled water and stained with three drops of 10 μg/ml phosphotungstic acid at pH 7.2. Samples were inspected in a transmission electron microscope (JEOL, 1010) at 60 kV. Images were obtained using an electron-sensitive CCD camera (Olympus, KeenView). For size determination, a cross-line carbon replica grid (2160 lines/mm) was used.

*FTIR*—FTIR was carried out using a Tensor 27 (Bruker) FTIR spectrophotometer equipped with a DTGS mid-infrared detector and a Golden Gate single reflection diamond attenuated total reflectance cell (Specac). Fibrils were dried on the attenuated total reflectance crystal using dry nitrogen. Attenuated total reflectance spectra were recorded from 4000 to 1000 $cm^{-1}$ using a nominal resolution of 2 $cm^{-1}$ and 128 accumulations. Resulting spectra were baseline-corrected, and interfering signals from $H_2O$ and $CO_2$ were removed using the atmospheric compensation filter in the OPUS version 5.5 system (Bruker).

*Circular Dichroism*—Circular dichroism (CD) spectra from 250 to 190 nm were collected on a Jasco J-810 spectropolarimeter using 0.2-nm steps, a scan speed of 50 nm/min, a bandwidth of 1 nm, and a response time of 1 s. A 1-mm quartz cuvette (Hellma) was used, and the temperature was kept constant at 20 °C with a thermostatically controlled cell holder (Jasco PTC 423S). The protein concentration was diluted to 0.1 mg/ml with double-distilled $H_2O$, and all spectra were baseline-corrected with respect to double-distilled $H_2O$. To improve the signal/noise ratio, five scans were averaged on each sample. Data points with a high tension voltage higher than 600 V were removed. The results were expressed as mean residue ellipticity.

*SPR*—FapC fibrils washed in double-distilled $H_2O$ were resuspended in HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 50 μM P20 (GE Healthcare), pH 7.4). Amyloid fibers were fragmented by sonication (80 Hz, 10 s) and centrifuged (14,000 × *g*, 30 s) to remove any large particles. 40 μl of centrate was mixed with 120 μl of 10 mM sodium acetate, pH 5.5, for the immobilization.

SPR experiments were performed at 25 °C using Biacore T-200 biosensor with research grade carboxymethylated (CM5S) sensor chips (Biacore, Uppsala, Sweden) with the exception of the binding of Congo Red, which was done in a Biacore 3000 instrument. Carboxymethylated CM5S chips were activated using 70 μl of 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl)carbodimide and 0.05 M *N*-hydroxysuccinimide in a 1:1 ratio. 100 μl of amyloids in sodium acetate solution (10 mM, pH 4.5) were injected over the activated surface, and an immobilization level of 8800 response units was reached. To confirm immobilization of the amyloids on the chip, solutions of 100 μM Congo Red (Sigma-Aldrich) or ThT (Sigma-Aldrich) in HBS-EP were injected over the immobilized amyloids for 45 s at a flow rate of 20 μl/min. Dissociation of the bound analytes was monitored for 1 min after the end of the injection. 20 μM stock solutions of analytes were prepared in HBS-EP with 5% DMSO. Serial 2-fold dilutions were performed in the same buffer to eight concentrations ranging from 0.16 to 20 μM. The chip surface was regenerated with HBS-EP for 100 s. All assays were performed in duplicate, and the averages are reported.

*QS Reporter Assays*—FapC fibrils and α-synuclein were suspended separately in buffer (10 mM HEPES, pH 7.4) to 4–5 μg/ml. 400 μl of each was then transferred to new 2.0-ml Eppendorf tubes, and QS molecules were added to a final concentration of 200 μM from a 25 mM stock in DMSO. The fibrils were incubated with the QS molecules overnight (22 °C, 200

rpm) and centrifuged (5 min, 1000 × *g*). The centrate was discarded, and the retentate was resuspended in 1 ml of buffer. This was repeated three times. Following the fourth centrifugation, the retentate was resuspended in 400 μl of buffer. This was performed for PQS and 3-oxo-C12-HSL.

*P. aeruginosa* PAO1 Δ*pqsC* $P_{pqsA}$::*gfp(ASV)* and Δ*lasI*Δ*rhlI* $P_{lasB}$::*gfp(ASV)* were grown overnight (37 °C, 200 rpm) in LB medium containing carbenicillin (100 μg/ml) and gentamicin (30 μg/ml), respectively. Overnight cultures of the reporter strains were mixed in ratios of 1:100 (v/v) with ABTGC medium (AB basal salts + 0.1% (w/v) thiamine + 0.2% (w/v) glucose + 0.5% (w/v) casamino acids). 180-μl aliquots of the diluted cultures were dispensed into the wells of a clear-bottom 96-well cell culture plate (Thermo Scientific). The volume in each well was then made up to 200 μl with buffer or the amyloid stocks before and after exposure to QS molecules diluted to 100, 50, 21, 7, and 4% of the original concentration. Plates were placed into a Tecan Infinite M200Pro (Switzerland) and incubated at 37 °C with shaking for 10 s prior to measurement, and fluorescence was detected every 20 min. At least three replicates were performed per assay.

*Electrical Characterization*—Electrical conductivity of the amyloid fibrils was characterized through a two-terminal electrode measurement. The fibrils were first dissolved in double-distilled $H_2O$ and drop-casted onto a 200-nm silicon nitride surface for uniform dispersion across the channel length. Silicon nitride was deposited through the plasma-enhanced chemical vapor method, and chromium was employed as the adhesion layer for the interdigitated gold electrodes (channel width, 1000 μm; channel length, 30 μm). This layer was fabricated through a series of lithography and electron beam deposition procedures. The substrates went through a standard cleaning process: acetone sonication (5 min), isopropyl alcohol sonication (5 min), deionized water sonication (5 min), and ultraviolet air plasma (2 min). 100 μl of dissolved fibrils were then drop-casted onto the cleaned substrate and allowed to dry overnight in the dark in ambient conditions to achieve a thin film through complete solvent evaporation. Current-voltage profiles were measured by a Keithley 4200 semiconductor characterization system interfaced to a Desert cryogenic probe station.

## RESULTS

*Preparation and Immobilization of Amyloid Fibers onto SPR Chip*—Native Fap fibrils purified from Fap-overexpressing *P. aeruginosa* strains usually contain considerable amounts of non-protein contaminants, such as extracellular polysaccharides. These contaminants hinder determination of the specific binding affinity between metabolites and the Fap fibrils. *In vitro* formed fibrils derived from recombinant FapC are clean of contaminants, but their structure may not be the same as the native amyloid structure due to the polymorphism associated with non-constrained fibril assembly (35).

In order to obtain clean native folded PAO1 Fap fibrils for binding studies, we took advantage of the autocatalytic nature of amyloid fibrils (36). Fibrillation kinetics of recombinant PAO1 FapC were greatly enhanced in the presence of 5% sonicated native PAO1 Fap fibrils, ensuring transmission of the native structure to the *in vitro* formed fibrils (Fig. 1*A*). The formed fibrils were then sonicated and used as seeds for another *in vitro* fibrillation in order to dilute contaminants originating from the native fibril seeds. This process was repeated to produce third generation PAO1 FapC fibrils.

The amyloid structure of the produced fibrils was confirmed by FTIR by the presence of the major amyloid characteristic peak between 1615 and 1630 $cm^{-1}$ (37) (Fig. 1*B*). The amyloid structure was furthermore supported by the circular dichroism (CD) spectra of the same fibrils, which showed a clear β-sheet signature (38) (Fig. 1*C*). Transmission electron microscopy confirmed the presence of clean, homogeneous fibrils similar to the native fibrils described previously for *P. aeruginosa* PAO1 (9) (Fig. 1*D*). SDS-PAGE of the produced fibrils confirmed that all monomers were converted into amyloids (data not shown).

The third generation FapC fibrils were fragmented by sonication and immobilized onto CM5S sensor chips for binding studies using amine-coupling chemistry. Congo Red and ThT bind to amyloids and can be used as diagnostic indicators of amyloidosis (39). Therefore, to confirm FapC fibril immobilization on the chip surface, the binding responses of Congo Red and ThT were followed. The sensorgram for Congo Red (Fig. 2*A*) clearly shows an association and a dissociation response. For ThT, on the other hand (Fig. 2*B*), a box-shaped response was observed, which is typical for weak or transient interactions. A concentration dependence of the response unit value following ThT injection confirmed that the amyloids had bound ThT (Fig. 2*C*). The equilibrium dissociation constant ($K_D$) for this ThT binding to amyloids was close to 100 μM, which is consistent with ThT acting as a transient affinity binding compound, as noted by Kawatake *et al.* (40). The binding responses of both Congo Red and ThT to the *Pseudomonas* amyloids were similar to those reported for Congo Red and ThT to amyloid prion proteins (40). These results indicate that amyloids had been immobilized successfully on the CM5S chip.

*QS Molecules Display Transient Binding to FapC Fibrils*—The binding affinities of the QS molecules pyocyanin, PQS, and 3-oxo-C12-HSL to the FapC fibrils were measured at concentrations ranging from 0.03 to 10 μM (Fig. 3, *A*, *C*, and *E*). Due to the limited solubility of each of these molecules in SPR running buffers, it was not possible to exceed this concentration, although these are within the range of biological relevance (41, 42). As with the binding response for ThT, a concentration-dependent box-shaped sensorgram was observed for the interactions of pyocyanin with the FapC fibrils, displaying clear association following the onset of injection and then dissociation at cessation of injection. For PQS and 3-oxo-C12-HSL the response was less pronounced, and the association and dissociation responses were more protracted. The magnitude of change in RI depends on the thickness and refractive layer of the molecular layer (43). The greater absolute change in RI elicited by pyocyanin interactions with FapC fibrils could reflect only that colored molecules like pyocyanin alter RI by a greater amount. It is therefore important to look at the concentration dependence of the responses. Here there is clearly concentration dependence, demonstrating binding between all of these metabolites and the FapC fibrils.

The average maximum binding responses (in response units) were plotted against pyocyanin, PQS, and 3-oxo-C12-HSL con-

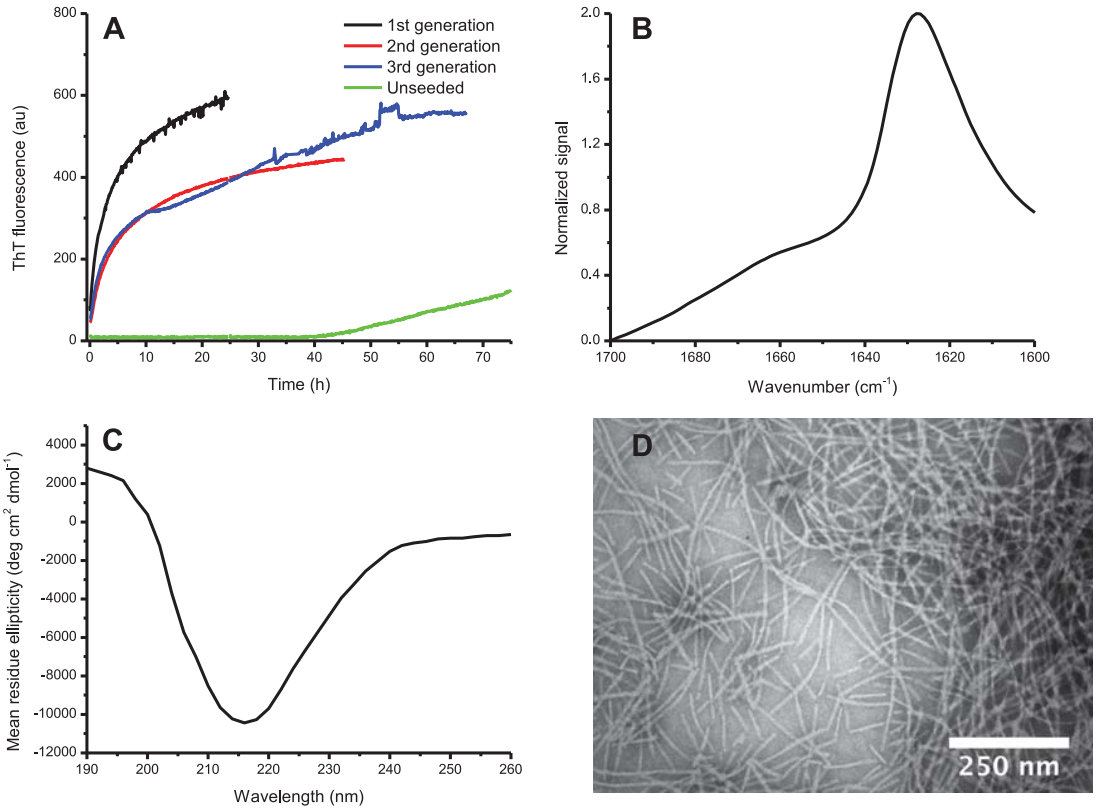


FIGURE 1. *A*, fibrillation kinetics of FapC unseeded or in the presence of 5% fibrils seeds visualized by ThT fluorescence. *B*, FTIR spectrum of third generation PAO1 FapC fibrils showing the amyloid characteristic peak between 1620 and 1630 $cm^{-1}$. *C*, circular dichroism spectrum of third generation PAO1 FapC fibrils showing a clear $\beta$-sheet signature with a single minimum at 217 nm. *D*, transmission electron microscopy images of third generation PAO1 FapC fibrils.

centrations, and the data were fitted to the steady state model of the evaluation software of Biacore T-200 (44) (Fig. 3, *B*, *D*, and *F*). $K_D$ values between the FapC fibrils and pyocyanin, PQS, and 3-oxo-C12-HSL were 400, 250, and 100 $\mu$M, respectively. These values and the box-shaped interaction kinetics indicate that they have transient binding to the fibrils (45). Metabolite binding was therefore similar to that of ThT, which is commonly used as a diagnostic tool for amyloids because of its altered spectral properties upon amyloid binding (46).

*Hydrophobicity Predisposes Metabolites to FapC Fibril Binding*—The three QS molecules screened are structurally different from each other and the positive control ThT (Fig. 4). To investigate whether features specific to *P. aeruginosa* metabolites predispose them to Fap fibril binding, binding responses to FapC fibrils of a range of biological molecules with different hydrophobicities and structures were determined (Figs. 5 and 6).

For the amino acids, the highest binding response was observed for L-tryptophan, which also has the highest hydrophobic index of all amino acids screened, as illustrated by the sensorgram (Fig. 5*A*) displaying gradual association and dissociation responses. Furthermore, average maximum binding response for L-tryptophan to FapC fibrils increased with molecular concentration ($K_D$ = 3 mM). Weaker binding responses were detected for the next most hydrophobic molecules, L-tyrosine (Fig. 5*C*) and L-histidine (Fig. 5*B*), with $K_D$ = 100 mM for both. No measurable binding response was observed for glutathione, L-glutamine, L-alanine, or L-glutamate (Fig. 6). They all have lower hydrophobic indices.

To investigate whether molecules functionally and structurally analogous to *P. aeruginosa* QS molecules, expressed by other organisms, bind with similar affinities to FapC fibrils, the binding response of riboflavin to FapC fibrils was screened. Like pyocyanin, riboflavin is hydrophobic with a central annulated pyrazine (Fig. 4*D*). As a soluble redox mediator, it performs a similar role for *Shewanella oneidensis* MR-1 as pyocyanin for *P. aeruginosa* (47). A binding response analogous to the one obtained for pyocyanin was observed for riboflavin (Fig. 5*D*), with strong response signal, box-shaped sensorgram, clear association and dissociation responses, and $K_D$ = 500 $\mu$M.

Binding therefore appears not to be specific for *P. aeruginosa* metabolites. Hydrophobicity, however, appears to predispose molecules to Fap fibril binding, as indicated by the fact that only the most hydrophobic amino acid tested achieved significant

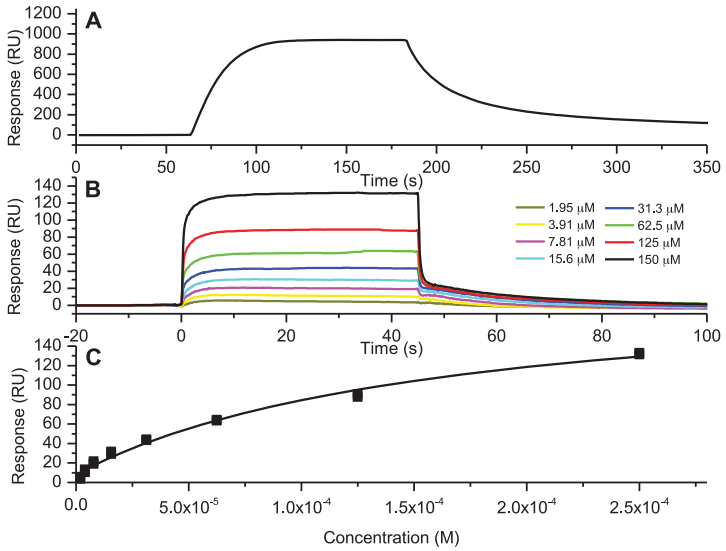


FIGURE 2. *A* and *B*, sensorgrams showing the interaction of Congo Red at 10 μM (*A*) and ThT at concentrations of 1.95–150 μM (*B*) with FapC fibrils. *C*, dose-response curve for the interaction between ThT and FapC fibrils. *RU*, response units.

binding affinities to the amyloid fibrils. This has implications not only for binding of *P. aeruginosa* QS molecules to amyloids but also retention of hydrophobic QS molecules and redox mediators expressed by Gram-negative bacteria more generally (48).

*Binding Responses of QS Molecules Are Characteristic of Fap Fibrils*—The specificity of QS molecule binding behavior for Fap fibrils *versus* non-amyloid proteins was tested using bovine serum albumin (BSA) and tobacco etch virus protease (*i.e.* His-tagged). Negligible binding to BSA and tobacco etch virus protease was recorded for pyocyanin, PQS, and 3-oxo-C12-HSL (data not shown).

Binding responses were screened for QS molecules to FapC monomers to determine whether fibrillation of FapC is a requirement for QS molecule binding. FapC monomers were immobilized onto the CM5S sensor within 30 min of desalting. In the absence of a fibril seed and within this short time period, the non-fibril state of the FapC monomers was probably preserved during immobilization (Fig. 1*A*). No measurable binding response was detected for any of the QS molecules screened against the FapC monomers (data not shown).

The binding responses of QS molecules to amyloid fibrils of the mammalian protein α-synuclein were then screened to determine whether their binding responses reflect a general feature of all amyloids and not just FapC fibrils from *Pseudomonas* (Fig. 7). Although pyocyanin was the only metabolite examined that bound to α-synuclein, this metabolite displayed a 10-fold lower affinity for the α-synuclein compared with that of binding to the FapC fibrils ($K_D$ = 4 mM). This shows that metabolite binding observed in this study is not a generic feature of all amyloid fibrils.

Fap fibrils can act as a reservoir for quorum-sensing compounds under non-quiescent conditions. The transient binding affinity of QS molecules to the FapC fibrils suggests that the fibrils are able to retain QS compounds under non-quiescent conditions and then act as a reservoir for these compounds. To provide evidence for this model, we employed *P. aeruginosa* reporter assays for PQS and 3-oxo-C12-HSL. The *pqsC* gene is required for the synthesis of the PQS precursor HHQ, and the *lasI* and *RhlI* genes are required for synthesis of 3-oxo-C12-HSL. The accompanying deletion strains are therefore unable to synthesize the two QS molecules on their own, and the fusion of the GFP gene to the *pqsABCDE* and *lasB* promoters allows PQS and 3-oxo-C12-HSL, respectively, to be monitored in terms of GFP fluorescence intensity (30, 32).

FapC fibrils were incubated separately with PQS and 3-oxo-C12-HSL for 24 h and then washed four times in buffer to remove unbound QS molecules, thus simulating non-quiescent conditions. The reporter strains were then challenged with various concentrations of the washed FapC fibrils, and the accessible QS molecules were detected directly by GFP fluorescence (Fig. 8).

When buffer was added as a negative control, a minimal increase in the GFP signal was recorded for both reporter strains, confirming their inability to synthesize the QS molecules. A clear increase was observed when standard solutions of PQS and 3-oxo-C12-HSL were added at the onset of the growth experiment, thus demonstrating the sensitivity of the reporter strain to the QS molecules (Fig. 8, *A* and *B*). The intensity of the GFP signal generated by both reporter strains normalized by cell density increased with concentration of QS-exposed FapC fibrils added even after four cycles of washing (Fig. 8, *C* and *D*). FapC fibrils not preincubated with QS molecules did not affect the GFP signal of the reporter strains (Fig. 8*E*), suggesting that the increase is due to the retention of QS molecules and not the fibrils themselves. The presence of

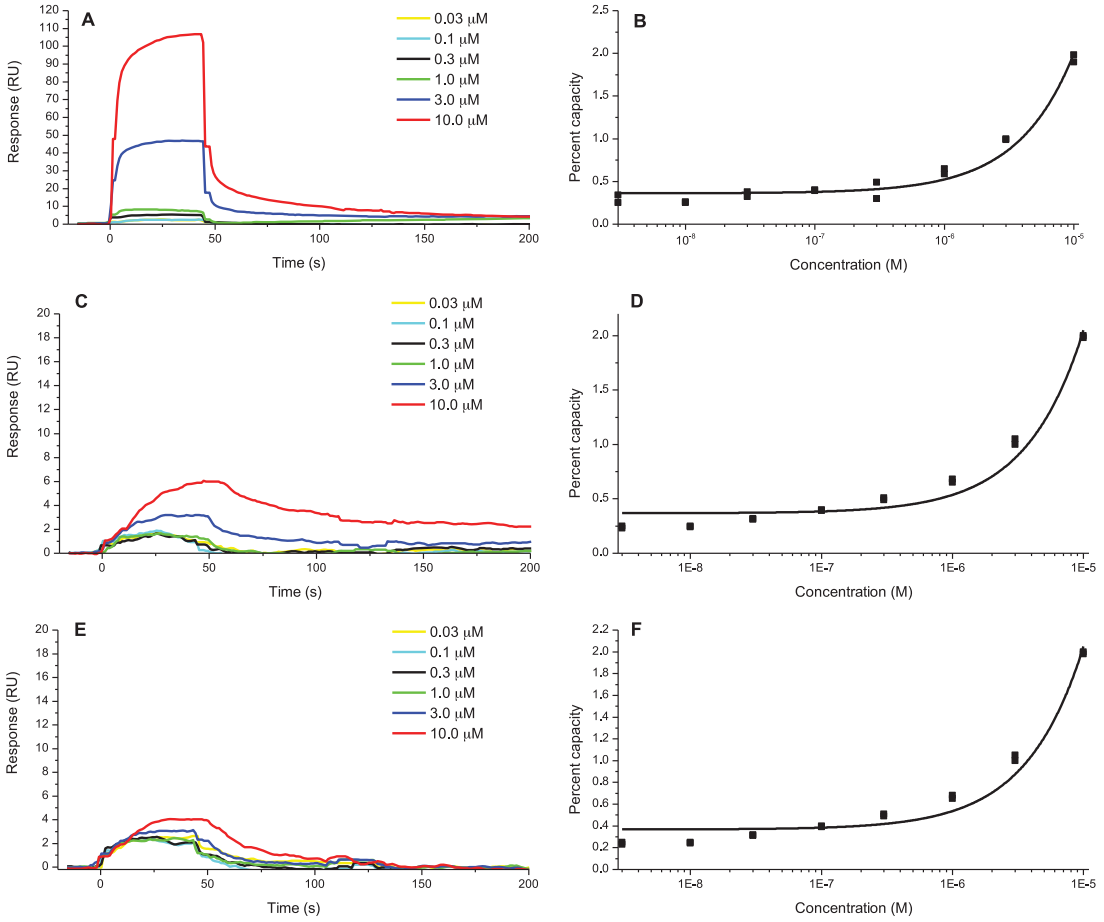


FIGURE 3. *A*, *C*, and *E*, sensorgrams showing the interaction of pyocyanin (*A*), PQS (*C*), and 3-oxo-C12-HSL (*E*) with FapC fibrils at concentrations ranging from 0.03 to 10 μM. *B*, *D*, and *F*, dose-response curves for the interaction between pyocyanin (*B*), PQS (*D*), and 3-oxo-C12-HSL (*F*) and FapC fibrils. Data are fit to a non-linear dose-response curve (*i.e.* sigmoidal), with 100% site occupation determined from extrapolation. *RU*, response units.

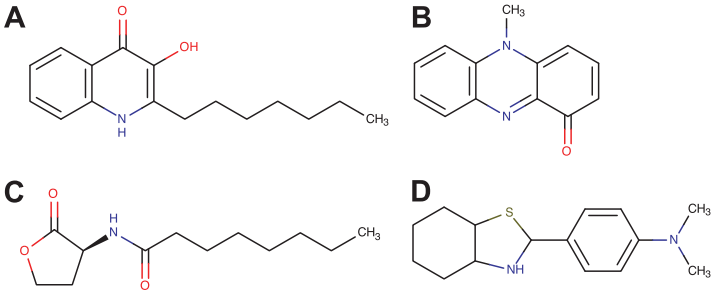


FIGURE 4. **Representative chemical structures of molecules belonging to three classes of *P. aeruginosa* quorum-sensing molecules, pyocyanin (*A*), PQS (*B*), and 3-oxo-C12-HSL (*C*), and thioflavin T (*D*).**

FapC fibrils therefore prevented the loss of these QS molecules by dilution, which is consistent with the observation by SPR that they bind to FapC fibrils.

On the other hand, no binding of either PQS or 3-oxo-C12-HSL to α-synuclein was observed by SPR. To validate SPR as a predictor of QS molecule retention, the serial dilution assays

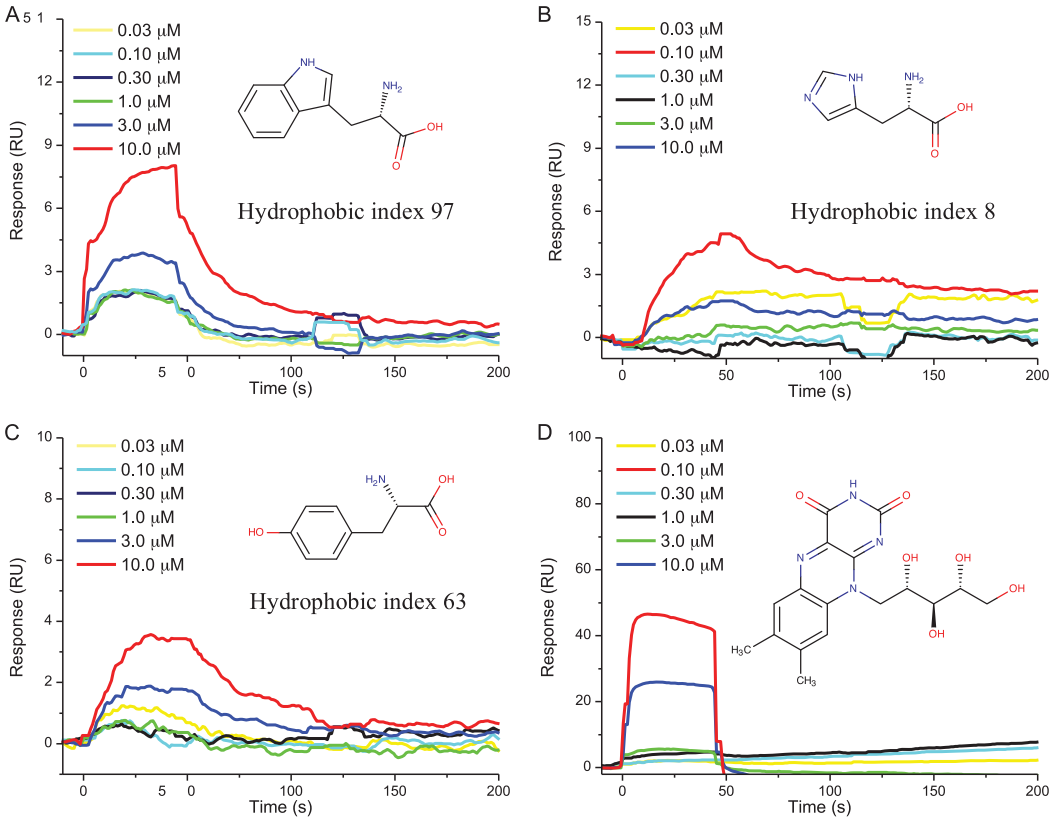


FIGURE 5. **Sensorgrams showing the interaction of L-tryptophan (*A*), L-histidine (*B*), L-tyrosine (*C*), and riboflavin (*D*) with FapC fibrils at concentrations ranging from 0.03 to 10 μM.** Chemical structures and hydrophobicity indices (for amino acids only) are presented in the *insets*. *RU*, response units.

were repeated for both QS molecules with α-synuclein (Fig. 8*F*). α-Synuclein preincubated with PQS and subjected to sequential washing failed to increase the GFP signal intensity of the reporter strains. The same was observed for α-synuclein preincubated with 3-oxo-C12-HSL (data not shown). Unlike FapC fibrils, the mammalian amyloid protein α-synuclein therefore could not prevent the loss of QS molecules by dilution, also consistent with the predictions by SPR.

*Pyocyanin Binding Can Enhance Electrical Conductivity of Fap Fibrils under Non-quiescent Conditions*—It has previously been shown that amyloid fibrils, such as those composed of the elastin-related polypeptide poly(Val-Gly-Gly-Leu-Gly), can conduct electrical signals (49). This conductivity was attributed to partial delocalization of peptide electrons caused by the high density of hydrogen bonding in β-sheet structures.

As a redox mediator, pyocyanin can enhance conductivity in biofilms (50). Binding of pyocyanin may therefore improve the conductive properties for Fap fibrils. To determine whether this is the case, FapC fibrils were incubated with or without pyocyanin and then washed four times in distilled water to remove unbound PQS and deposited by drop-casting onto interdigitated gold. Without exposure to pyocyanin, average current values of ~1 nA were recorded for the FapC fibrils, similar to that determined for elastin-related amyloid fibrils (49) but considerably lower than that measured for α-synuclein (~17 nA) (Fig. 9). Drop-casted pyocyanin onto the electrodes produced a maximum current of ~600 nA (Fig. 9). Preincubation of FapC fibrils and α-synuclein with pyocyanin elevated their conductivities to ~200 and 170 nA, respectively (Fig. 9), despite four cycles of washing with distilled water. This confirms the robust nature of the pyocyanin binding onto the amyloid fibrils. Additionally, current generation in the fibrils remained relatively stable following each scan.

## DISCUSSION

Direct and quantitative evidence is provided here that strongly suggests a function for Fap fibrils in binding extracellular metabolites, such as quorum-sensing molecules and redox mediators, with pyocyanin, PQS, and 3-oxo-C12-HSL used in this study to illustrate this. Pyocyanin is a direct determinant of *P. aeruginosa* virulence (51, 52) and enables the cells to maintain redox balance under anaerobic conditions (53). PQS protects against oxidative stress (54) and induces *lasB* and *rhlI*

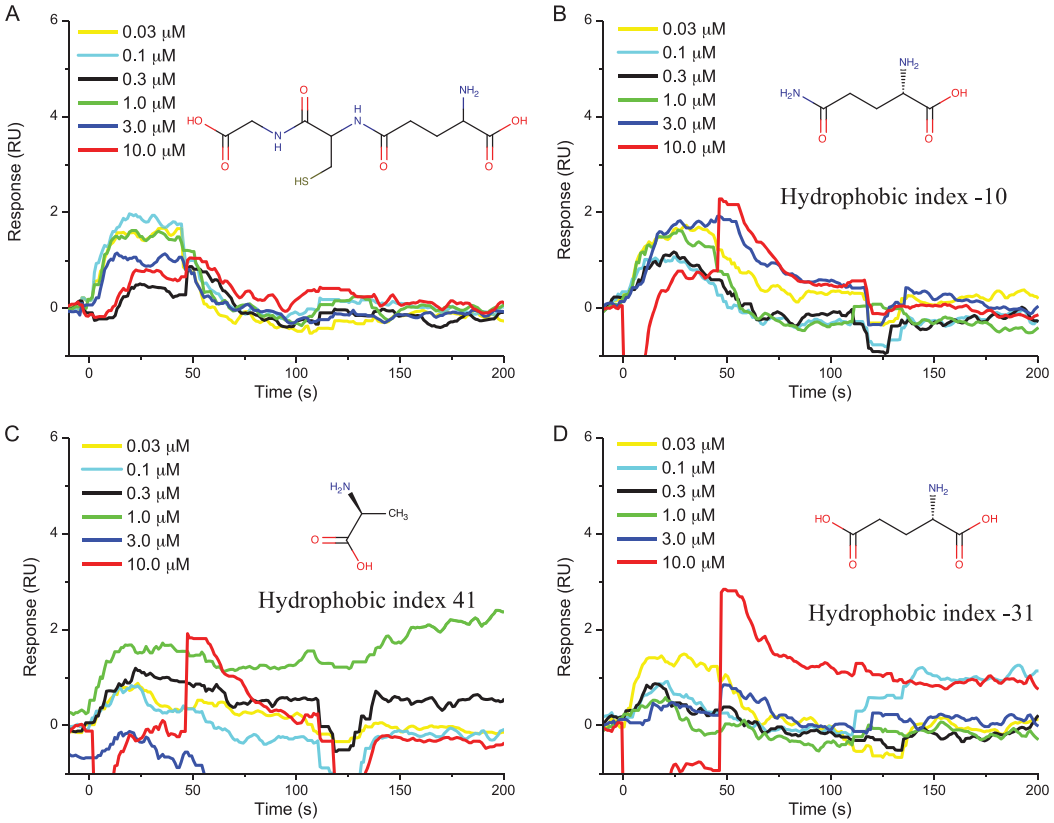


FIGURE 6. **Sensorgrams showing the non-interaction of L-glutathione (*A*), L-glutamine (*B*), L-alanine (*C*), and L-glutamate (*D*) with FapC fibrils at concentrations ranging from 30 nM to 10 μM.** Chemical structures and hydrophobicity indices (for amino acids only) are presented in the *insets*. *RU*, response units.

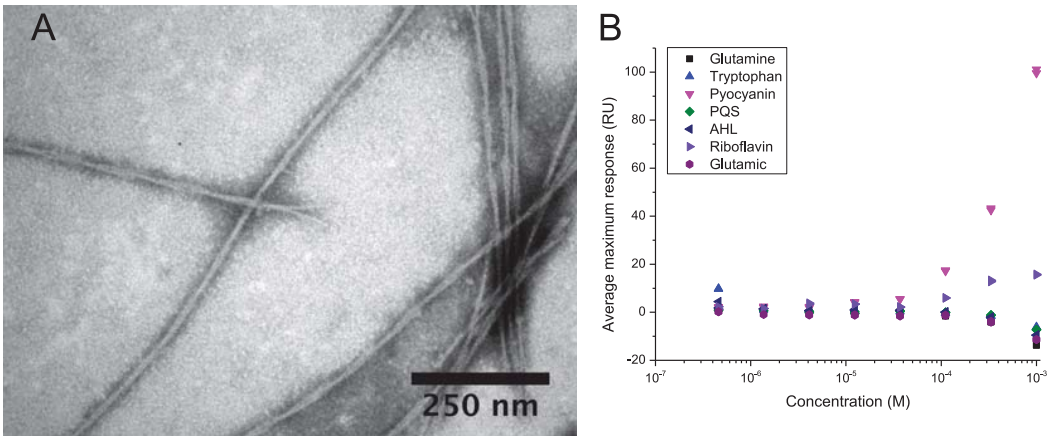


FIGURE 7. *A*, transmission electron microscopy image of α-synuclein fibrils; *B*, dose-response curves for the interaction between quorum-sensing molecules and control amino acids with α-synuclein fibrils. *RU*, response units.

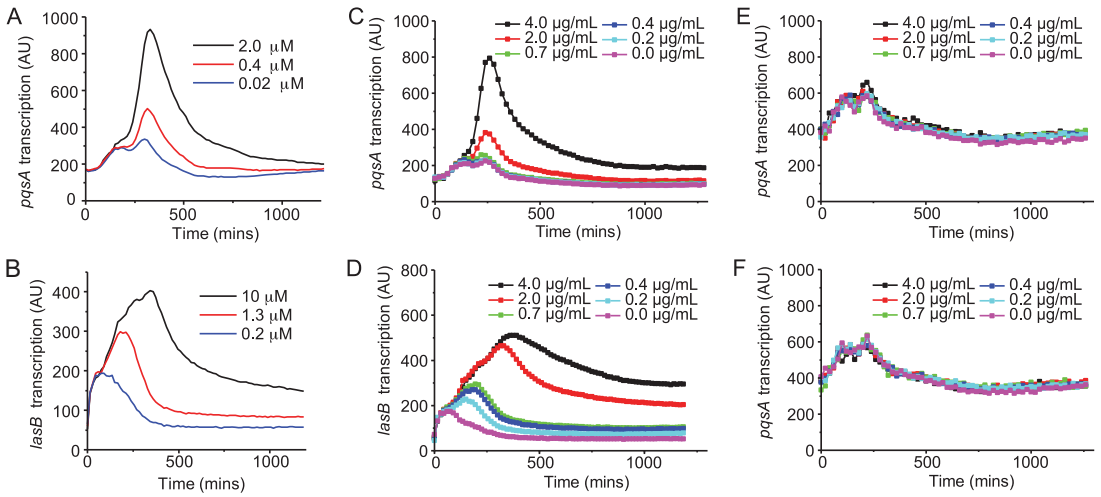


FIGURE 8. *A* and *C*, transcription of *pqsA* in a *P. aeruginosa pqsA::gfp(ASV)* reporter strain unable to synthesize PQS upon the addition of various concentrations of pure PQS (*A*) or PAO1 FapC fibrils (*C*) preincubated with PQS. *B* and *D*, transcription of *lasB* in a *P. aeruginosa lasB::gfp(ASV)* reporter strain unable to synthesize 3-oxo-C12-HSL upon the addition of various concentrations of pure 3-oxo-C12-HSL (*B*) or PAO1 FapC fibrils preincubated with 3-oxo-C12-HSL (*C*). *E* and *F*, transcription of *pqsA* in a *P. aeruginosa pqsA::gfp(ASV)* reporter strain upon the addition of various concentrations of FapC fibrils without PQS preincubation (*E*) and α-synuclein fibrils preincubated with PQS (*F*). All fibrils were washed four times with buffer to remove unbound QS molecules.

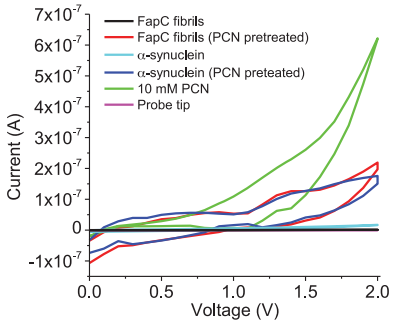


FIGURE 9. **Current-voltage (*I-V*) characteristics of PAO1 FapC and α-synuclein fibrils with and without preincubation with pyocyanin (*PCN*).** A 10 mM PCN solution following deposition and evaporation and the probe tip alone were used as positive and negative controls, respectively. All fibrils were washed four times with buffer to remove unbound QS molecules.

expression to disrupt host immune responses (55, 56). Interactions between such clinically important agents and a constituent of the extracellular matrix of *P. aeruginosa* biofilms (9) are implicitly significant and show that Fap fibrils can facilitate the retention and release of extracellular metabolites when required. Thus, molecular retention needs to be added to the properties assigned to bacterial amyloids (*i.e.* their ability to facilitate interactions with host proteins, through curli (2, 57), and to contribute to biofilm formation and adhesion, as is the case for TasA and Fap) (8, 58).

There appears to be growing evidence that matrix components have multiple functions, as demonstrated by extracellular DNA, which are involved in the chelation of cations and antibiotic resistance (59), horizontal gene transfer (60), and matrix self-organization (61). Multiple biological roles are also seen for the Psl exopolysaccharide of *P. aeruginosa*, which, besides being a structural component of biofilms (62), also displays regulatory functions (63). Whether such effects are primary or secondary effects of the EPS synthesis remain to be established. For example, the ability of amyloids to capture signaling molecules suggests an indirect role in mediating cell function. Nonetheless, as with amyloid fibrils, synthesis of these additional matrix components has a range of phenotypic consequences, and indeed products and traits with multiple functions of individual components or products are commonplace adaptive features of bacteria and may be necessary for resilience.

The task of assigning motive for EPS synthesis and understanding whether secondary effects are deliberate strategies of the bacteria or unintended consequences is therefore complicated. Performing direct and quantitative functional assignment on a matrix constituent does not address the question of motive. It does, however, provide key information in the causal chain between an EPS and phenotype. Similar to observations made for the exopolysaccharide Psl, a regulatory role was recently also demonstrated for Fap fibrils on the basis that they elicit major changes to the proteome of *P. aeruginosa* (64). Fap proteins are not expected to have any direct regulatory capacities, and the proteomic changes were postulated as being secondary effects resulting from interference by Fap fibrils of quorum-sensing signaling (64). By means of single molecular functional analysis on FapC fibrils, we can therefore hone in specifically on these interactions between matrix constituents and QS molecules and provide further support to the hypothesis that such interactions have important consequences. Such QS molecules are highly concentrated in biofilms (65), and we demonstrate in this work that as long as these QS molecules are expressed in a biofilm containing FapC fibrils, the fibrils will serve to prevent their loss from dilution under non-quiescent conditions.

Additionally, demonstrating an interdependence between matrix constituents and QS molecules highlights a shortcoming of a popular approach toward allocating a functional assignment of EPS in biofilms, which is to generate EPS knockdown mutants and then indirectly assign functions by observing any phenotypic differences (12, 66). As demonstrated here, changing the EPS balance will set off a cascade of secondary effects by, for example, affecting local concentrations of metabolites in the cell environment. It is thus important to employ a method for direct functional assignment for individual EPS.

Transient interactions are common and often advantageous in biological systems. In dense biofilm matrices, there are many binding sites within close proximity to each other. Rapid association and dissociation kinetics, as demonstrated here, allow for greater bioavailability (45). Rapidly dissociated QS molecules will quickly rebind to the nearest site with parallel and serial bindings that can augment binding strength (67). This is the basis of improving efficacy of many drugs and small inhibitors (68). FapC fibrils are therefore likely to be important in turbulent and competitive environments commonly encountered in non-laboratory environments (*e.g.* wastewater treatment plants and acute respiratory infections). The sorptive properties of amyloids ensure that they would minimize the loss or interception by other bacterial cells of molecules required to transfer information between individual producing cells. Synthesis of amyloids could therefore minimize the loss of metabolites to other bacteria as well as enable *P. aeruginosa* to capture and store metabolites produced by other bacteria. Amyloid binding may also be significant for siderophores and a range of other public good molecules.

Unlike the dissimilatory metal reducers *Geobacter* and *Shewanella*, conductivity is not considered an essential property of *P. aeruginosa* biofilms any more than it is considered a necessary function of pyocyanin (53, 69). However, this study illustrates quantitatively how an EPS might contribute to extracellular electron transfer. Researchers are still divided on how cells might achieve extracellular electron conduction, with metallic-like conductivity (70) and superexchange, in which electrons are conducted by a succession of electron transfer reactions (71), identified as two possible mechanisms. Enhanced conductivity due to reversible binding of redox-active agents along the length of the EPS is therefore consistent with the superexchange explanation.

Furthermore, although extracellular polymeric substances are thought to serve an important function in retaining molecules and proteins, both endogenous and exogenous, in biofilms (27), to the authors' best knowledge, direct binding of a metabolite to any individual EPS component has hitherto not been quantified. Das *et al.* (72) demonstrated pyocyanin binding to extracellular DNA, based on the displacement of ethidium bromide from DNA following pyocyanin addition. SPR was used here not only to demonstrate binding between clinically relevant metabolites and an EPS component but furthermore to quantify this interaction and describe binding specificity. Quantification of binding affinities is essential in establishing and assessing the significance of metabolite-EPS interactions.

Establishing that bacterial amyloids bind QS molecules in an orderly manner suggests a function for them as reservoirs for information transfer. These results unveil a mechanism by which biofilm communities can manage information transfer in non-quiescent and competitive environments. Amyloid expression may thus confer a competitive advantage on some populations under such conditions. It remains to be seen whether amyloids are matrix components of general benefit in biofilms by their ability to retain all signaling molecules or only those expressed by certain strains. A better understanding of binding specificity could lead to biofilm mitigation or management strategies that target metabolite binding sites on matrix components like amyloid polymers.

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# APPENDICES

# Appendix A. Pilot-scale MBR plant overview

In the EcoDesign project, a pilot-scale MBR was built at the conventional full-scale plant at Aalborg West, Denmark. The pilot plant served as sampling site for MBR activated sludge and was used in different experiments and in many interdisciplinary projects, including this PhD project. The setup is described in detail in the study of Ziegler et al., (2016), however, in brief: the influent wastewater came from the same primary settling tank as the full-scale plant, entering an anoxic/denitrification (2 $m^3$) tank and going to an aerobic/nitrification (2 $m^3$) tank through gravitation. The influent flow was 0.5 $m^3$/h and was operated with a mixed liquid suspended solids (MLSS) concentration of 3.6–5.4 kg/$m^3$. It was configured with return sludge sidestream hydrolysis (RSS), which is very common in Danish plants and ensures good enhanced biological phosphorus removal (EBPR) (Mielczarek et al., 2013). In the aerobic tank, a membrane cassette with twenty flat-sheet MFP2 membranes (polypropylene, Alfa Laval) with a surface area of 40 $m^2$ and an average pore size of 0.2 μm was submerged. A mini-membrane cassette with seven membranes with a 0.225 $m^2$ surface area was used for sampling and was coupled to the large membrane cassette to ensure identical transmembrane pressures (TMP). A constant TMP of 0.003 MPa (or 30 mbar) was maintained across the membrane module, and filtration was carried out intermittently in cycles of 8 min filtration and 2 min relaxation. The reactor flow was constant around 0.69–0.71$m^3$/h. The membrane modules were scoured from beneath, where bubble diffusers were positioned to supply oxygen to the mixed liquid and to clean the surface of the membranes. A compressor was used to control the airflow rate. TMP, dissolved oxygen concentration and permeate flux were monitored online. Figure A1 gives an overview of the system.

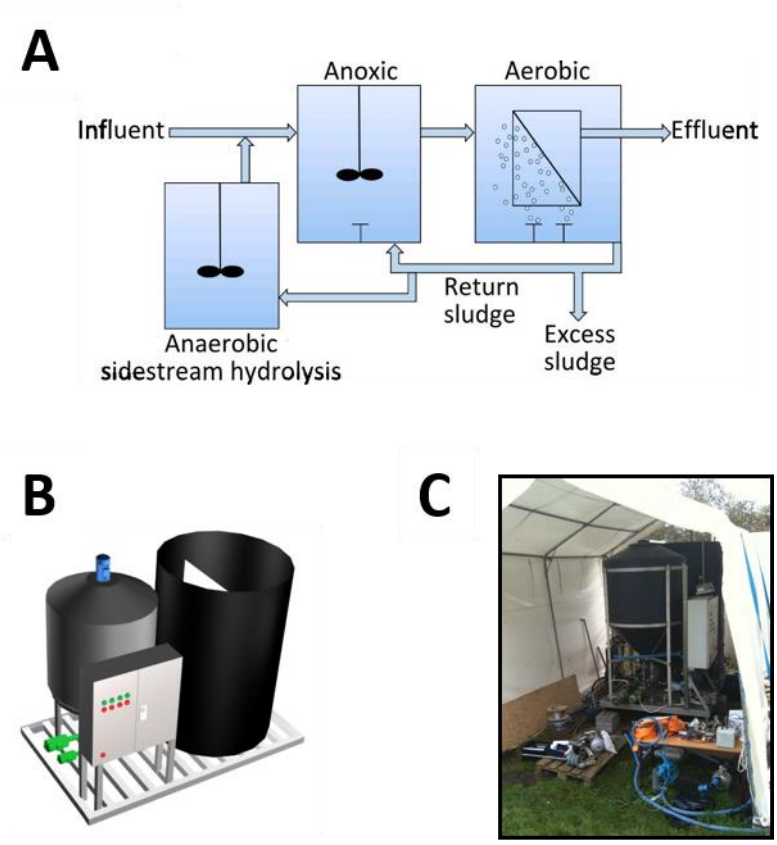


*Figure A1 – A) Diagram of pilot-scale MBR plant at Aalborg West WWTP, B) 3D model, and C) photo on site.*

# Appendix B. TMP step test method

In order to study the filtration properties of sludge and suspensions, a fully automated filtration test system, the so-called Aalborg Filtration Property Analyzer (AaFPA), was used to characterise fouling layer compressibility and its effect on hydraulic resistance during short-term filtrations. The method was previously described by Jørgensen, Bugge, et al., (2017). The filtration system consists of a flat sheet system with aerated membranes (Figure B1). The total volume of the reactor was 5 L and the membrane area was 84 $cm^2$ of an Alfa Laval MFP2 flat sheet membrane. The airflow was kept constant at 7.5 L/min and the temperature was kept stable at around 22 °C. The permeate flux was calculated by weighing the permeate online, using a highly sensitive scale and dividing by the membrane area. The transmembrane pressure (TMP) was adjustable and controlled by the water level difference between the bioreactor and the permeate beaker.

A TMP step program was applied for the filtration experiments and the program designed to increase TMP with 2 kPa for each step, which gives the pressures 1, 3, 5, 7, 9, 11, and 13 kPa (Figure B2). The filtration phase was set to one hour to be able to obtain a steady-state level of flux and to ensure removal of any reversible fouling from the preceding filtration step. The TMP step program ends with a post-filtration for one hour at 1 kPa to check for irreversible deposition of foulants.

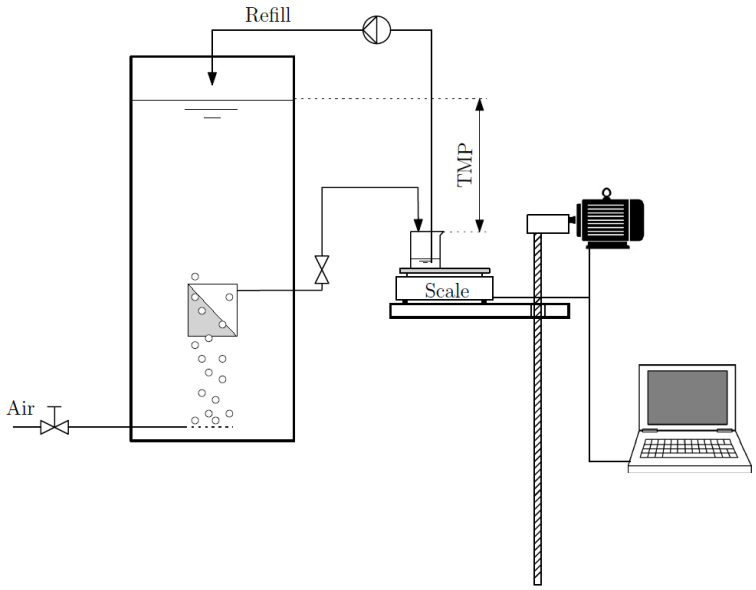


*Figure B1 - Laboratory-scale air-scoured flat-sheet MBR system. Aalborg Filtration Property Analyzer (AaFPA).*

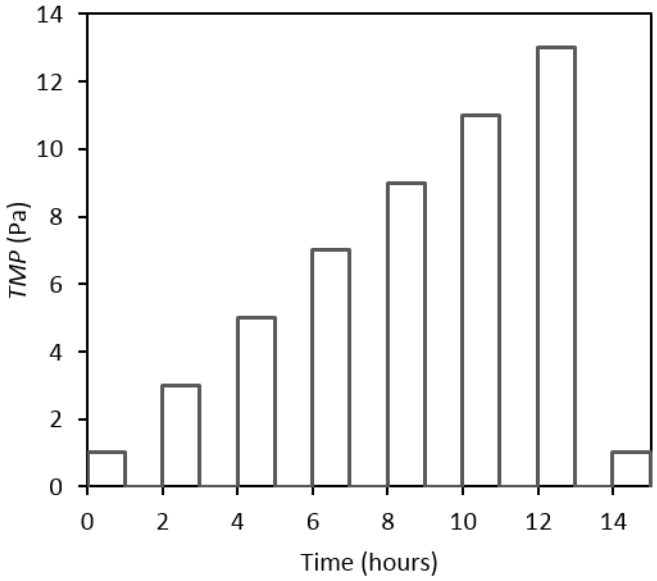


*Figure B2 - TMP step protocol for filtration experiments.*

Both the model and theory of cake build-up and compression have previously been described by Bugge et al., (2012). This is based on a simple relationship between flux ($J$) and applied transmembrane pressure ($TMP$) derived from Darcy's law, where $\mu$ denotes liquid viscosity, $R_m$ the resistance of the membrane, and $R_f$ the resistance resulting from the fouling layer or cake layer:

$$J = \frac{TMP}{\mu \cdot (R_m + R_f)}$$

The hydraulic resistance of the deposited cake ($R_f$) may be expressed by the specific resistance to filtration ($\alpha$) as:

$$R_f = \alpha \cdot \omega$$

where $\omega$ is the filter cake mass per area of membrane. For biological materials, there is a linear relation of specific resistance and pressure:

$$\alpha = \alpha_0 \left(1 + \frac{TMP}{P_\alpha}\right)$$

where $\alpha_0$ is the specific resistance of the cake at zero pressure and $P_\alpha$ is the pressure needed to double the specific resistance and takes effects from compressibility into account. A high $P_\alpha$ indicates low compressibility and vice versa. If compressibility is high, increasing TMP will automatically cause a higher $\alpha$ as ($\alpha_0 \cdot$ TMP/$P_\alpha$) increase proportionally.

The average specific resistance at zero pressure ($\alpha_0$) and its pressure dependence ($P_\alpha$) can be determined by extrapolation of data from a filtration step test, as illustrated in Figure B3.

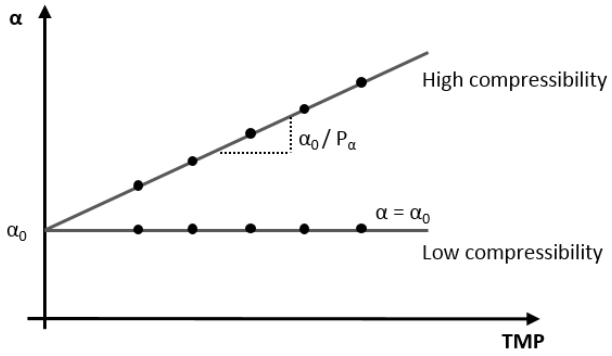


*Figure B3 - Specific resistance and TMP.*

The cake build-up on the membrane surface can be explained by sludge particles that are exposed to different transport mechanisms. The mechanisms include gravitation, air scouring, permeate drag, and diffusion (Figure B4). Due to shear, gravitation is negligible. Permeate drag is the main force responsible for transport towards the membrane. The transport mechanism depends on the size of the particles, whereas only small particles are affected by diffusion within the boundary layer. The main force responsible for transport away from the membrane is air scouring. The effect of air scouring decreases close to the membrane due to the diffusive boundary layer. Particles that are larger than the boundary layer, such as sludge flocs, will be subjected to air scouring.

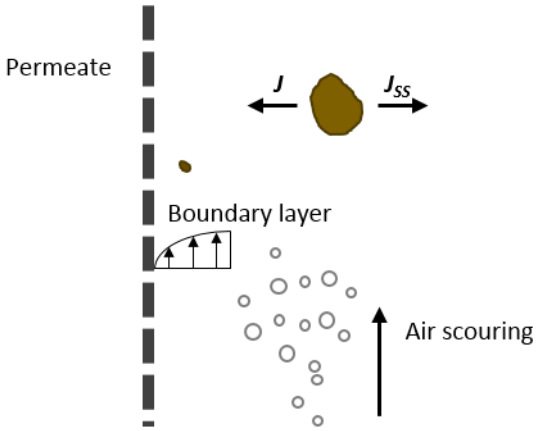


*Figure B4 - Transport mechanisms in cake build-up.*

Based on these mechanisms, the cake build-up is modelled by:

$$\frac{d\omega}{dt} = (J - J_{SS}) \cdot c_b$$

Where $J$ is permeate flux, $J_{SS}$ is the back transport, and $c_b$ is the bulk sludge concentration. However, as it is known that $J_{SS}$ varies according to particle size, it is probably an oversimplification to use the bulk sludge concentration as $c_b$.

Another way to describe the fouling mechanism is by different terms relating to the performance of the system, e.g. critical flux ($J_{crit}$), steady-state flux ($J_{SS}$), and limiting flux ($J_{lim}$) (Jørgensen et al., 2014). The steady-state flux is reached when the flux stabilises, and it can be determined from the experimental flux curves as illustrated in Figure B5. Typically, a pronounced decrease in initial flux is observed, which levels off and approaches the steady-state flux. The critical and limiting flux can be found

from the steady-state fluxes plotted against TMP (Figure B5). $J_{crit}$ is pressure independent and is the flux above which the flux declines over time. Hence, the limiting flux is the highest steady-state flux that can be reached. It is of special interest for MBR operators as the MBR should be operated at a flux below the "critical flux" in order to minimise membrane fouling and maintain sustainable permeability. A high limiting flux represents low fouling propensity as it is also a measure of back transport of foulants away from the membrane due to air scouring.

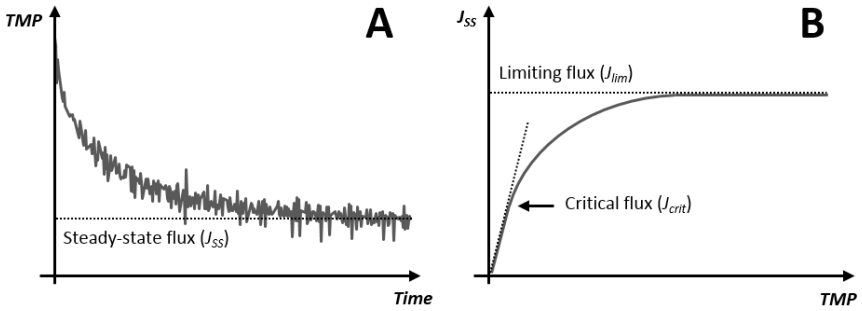


*Figure B5 – Steady-state flux. A) Experimental flux data and indication of steady-state flux ($J_{SS}$). B) Steady-state flux as a function of TMP with indications of limiting flux and critical flux.*

**List of symbols**

| | |
|---|---|
| $C_b$ | Bulk concentration (kg/m$^3$) |
| $J$ | Permeate flux (m/s) |
| $J_{SS}$ | Steady-state flux (m/s) |
| $J_{lim}$ | Limiting flux (m/s) |
| $J_{crit}$ | Critical flux (m/s) |
| $TMP$ | Transmembrane pressure (Pa) |
| $P_\alpha$ | Characteristic pressure (Pa) |
| $R_f$ | Fouling resistance (m$^{-1}$) |
| $R_m$ | Membrane resistance (m$^{-1}$) |

**Greek symbols**

| | |
|---|---|
| $\alpha$ | Average specific resistance (m/kg) |
| $\alpha_0$ | Average specific resistance at zero pressure (m/kg) |
| $\mu$ | Permease viscosity (Pa s) |
| $\omega$ | Specific cake mass (kg/m$^2$) |

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